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CAROLO-WILHELMINA
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Regulatory and metabolic adaptation of *Pseudomonas aeruginosa* to changes in oxygen tension and growth phase

Von der Fakultät für Lebenswissenschaften
der Technischen Universität Carolo-Wilhelmina
zu Braunschweig
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Doktorin der Naturwissenschaften
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von Beatrice Benkert
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Benkert, B., Quäck, N., Schreiber, K., Jaensch, L., Jahn, D., and Schobert, M. (2008) Nitrate-responsive NarX-NarL represses arginine-mediated induction of the *Pseudomonas aeruginosa* arginine fermentation *arcDABC* operon. *Microbiology* 154, 3053-3060.

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MEINER MUTTER

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ABBREVIATIONS

2D	two-dimensional
A_λ	absorption at wavelength λ in nm
ADI	arginine deiminase
AHL	acyl-homoserine lactone(s)
Ap	ampicillin
APS	ammonium persulfate
AMP	adenosine monophosphate
Anr	anaerobic regulation of arginine deiminase and nitrate reduction
Arg	arginine
ATP	adenosine triphosphate
bp	base pair(s)
C	Celsius (℃)
cDNA	copy DNA
CF	cystic fibrosis
Cm	chloramphenicol
CM	cytoplasmatic membrane
Crp	cAMP receptor protein
CFTR	cystic fibrosis transmembrane conductance regulator
CWW	cell wet weight
DIG	digoxygenin
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
(d)dNTP	(di)deoxyribonucleotide triphosphate
(d)dUTP	(di)deoxyuridine triphosphate
Dnr	dissimilatory nitrate respiration regulator
ds	downstream
dsDNA	double stranded DNA
EDTA	ethylenediaminetetraacetic acid
e. g.	*exempli gratia* (for example)
ESI	electrospray ionization
et al.	*et alteri* (and others)
EtOH	ethanol
FBA	flux balance analysis
fig.	figure
Fnr	fumarate and nitrate reduction
for	forward
g	centrifugation: earth gravity (x g); weight: gram (g)
GC	gas chromatography
Gm	gentamicin

h	hour
H_2O_{deion}	deionized water
IM	inner membrane
kb	kilo base pair(s)
KEGG	Kyoto Encyclopedia of Genes and Genomes
l	liter
LB	Luria Bertani
LC	liquid chromatography
m	milli
M	molar (mol l^{-1})
µ	micro
MALDI-TOF	matrix assisted laser desorption/ionization - time of flight
Mb	mega base pair(s)
MCS	multiple cloning site
MetOH	methanol
min	minute
MOPS	3-(N-morpholino) propanesulfonic acid
MS	mass spectrometry
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
MU	Miller units
n	nano
NAD(P)	nicotine adenine dinucleotide (phosphate)
NMR	nuclear magnetic resonance spectroscopy
nt	nucleotide(s)
OD_λ	optical density at wavelength λ in nm
OM	outer membrane
OMP	outer membrane protein(s)
ONPG	o-nitrophenyl-β-D-galactopyranoside
ORF	open reading frame(s)
ori	origin of replication
PAGE	polyacrylamide gel electrophoresis
PCI	phenol-chloroform-isoamylalcohol
PCR	polymerase chain reaction(s)
PMF	proton motive force
PRODORIC	Prokaryotic database of gene regulation
QS	Quorum sensing
RBS	ribosome binding side
rev	reverse
RMA	robust multichip average
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
RT	room temperature
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
sec	second
ssDNA	single stranded DNA
t	time
tab.	table
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEMED	tetramethylethylenediamine
Tc	tetracycline
T_M	hybridization temperature
Tris	tris(hydroxymethyl)aminomethane
U	unit
UMP	uridine monophosphate
us	upstream
UV	ultraviolet
V	volt
v	volume
vs.	versus
v/v	volume per volume
w/v	weight per volume
wt	wild type

1 INTRODUCTION

1.1 *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa is a Gram-negative motile and rod-shaped bacterium belonging to the gamma group of proteobacteria. It is a ubiquitous organism found in a wide variety of ecological niches like soil, marshes and aquatic habitats as well as plants, animals and humans (Bodey *et al.*, 1983).

The *P. aeruginosa* genome is one of the largest bacterial genomes currently sequenced with a size of 6.3 mega base pairs (Mb) and 5,570 predicted open reading frames (ORF) (Stover *et al.*, 2000). More than 40 % of the genome consists of hypothetical and conserved hypothetical ORFs with unknown function (Stover *et al.*, 2000; Winsor *et al.*, 2005). Compared to other bacteria, the *P. aeruginosa* genome consists of more regulatory genes encoding two-component systems, transcriptional regulators and transport systems reflecting its genetic complexity (Stover *et al.*, 2000). Over 550 predicted transcriptional regulators and more than 60 putative two-component systems were identified in *P. aeruginosa* allowing a rapid adaptation to environmental changes via controlled gene expression (Potvin *et al.*, 2008; Rodrigue *et al.*, 2000). About 150 predicted outer membrane proteins (OMP) are involved in iron uptake, antibiotic export and secretion of virulence factors essential for pathogenicity of *P. aeruginosa* (Stover *et al.*, 2000). Consistent with the high number of about 200 of cytoplasmic membrane transport systems importing nutrients and other molecules, *P. aeruginosa* is able to degrade various carbon sources, *e. g.* carbonic acids, sugars, fatty acids, alcohols, polyalcohols, glycols, aromatic compounds, amines and amino acids (Stover *et al.*, 2000).

P. aeruginosa is found as free-living planktonic cells or as sessile biofilms. Biofilms cause a variety of problems in industrial settings, *e. g.* drinking water systems. Most importantly, biofilms are associated with many chronic infections in humans (Costerton *et al.*, 1999). Biofilm formation and in addition the production of a large arsenal of virulence factors depend on a cell-to-cell communication system called quorum sensing and are responsible for maximum virulence of *P. aeruginosa* (Davies *et al.*, 1998).

All factors together may explain the successful adaptation of *P. aeruginosa* to diverse ecological niches.

1.1.1 *Pseudomonas aeruginosa* as opportunistic human pathogen

Pseudomonas aeruginosa is an opportunistic human pathogen commonly found in nosocomial infections. It is responsible for pneumonia, urinary tract infections and wound infections of hospitalized patients and causes infections of immunocompromised people, *e. g.* AIDS and cancer patients (Van Delden and Iglewski, 1998). Moreover, *P. aeruginosa* is the predominant lung pathogen of patients suffering from cystic fibrosis (CF) and responsible for high rates of mortality (Govan and Deretic, 1996).

1.1.1.1 Cystic fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder caused by a defective CF transmembrane conductance regulator (CFTR), which normally transports Cl^- from the basolateral to the apical surface of the airway epithelium (Riordan *et al.*, 1989). A defective CFTR leads to strong Na^+, Cl^- and water absorption and thus to accumulation of dehydrated secretions on the apical surface. The CF lung is characterized by such abnormal mucus, which provides good conditions for the colonization by *P. aeruginosa* and other opportunistic pathogens (Hassett *et al.*, 2002). The ability of *P. aeruginosa* to form biofilm-like microcolonies embedded in the mucus significantly increases the resistance to the human immune response and to antibiotic therapies (Costerton *et al.*, 1999; Singh *et al.*, 2000). Within the mucus low oxygen to anaerobic regions were observed implying that the bacteria switch from aerobic to anaerobic growth (Hassett *et al.*, 2002; Worlitzsch *et al.*, 2002). Figure (fig.) 1.1 shows the proposed infection process of *P. aeruginosa* in the CF lung (Williams *et al.*, 2007; Worlitzsch *et al.*, 2002)

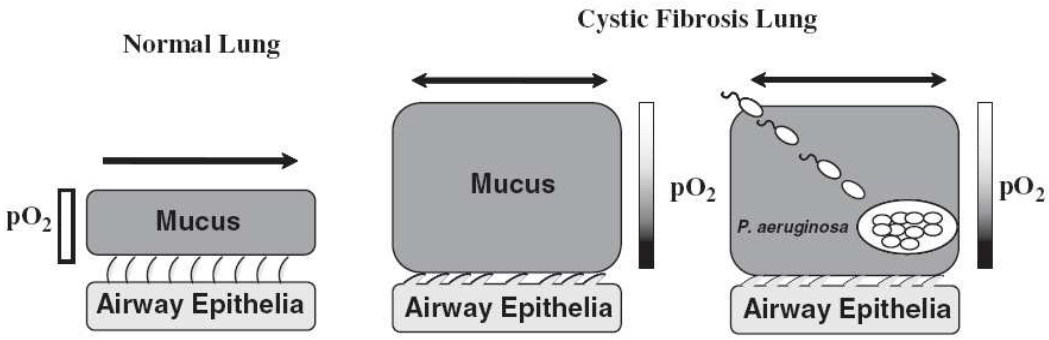


Figure 1.1: Schematic model of the infection process of *P. aeruginosa* in the CF lung, adapted from (Williams *et al.*, 2007).

In healthy lungs the thin mucus is cleared by the action of cilia on the epithelia cells. In contrast, in the CF lung the cilia are blocked by the dehydrated thick mucus caused by the defective CFTR. After penetration, *P. aeruginosa* grows as biofilm-like microcolonies inside the mucus. Within the mucus oxygen gets limited and bacteria adapt to anaerobic conditions.

1.2 *PSEUDOMONAS AERUGINOSA* ENERGY METABOLISM

P. aeruginosa shows a wide diversity in energy generation pathways and is capable to use different carbon and energy sources as mentioned above. In contrast to bacteria preferring the Embden-Meyerhof pathway, *P. aeruginosa* uses the Entner-Doudoroff pathway for the oxidation of glucose (Lessie and Phibbs, 1984). However, instead of glucose, *P. aeruginosa* favors organic acids, especially products of the citrate cycle as carbon and energy source (Palleroni, 1992). In agreement, the *P. aeruginosa* genome lacks most of sugar transporters commonly found in other bacteria (Stover *et al.*, 2000).

Energy generation in form of adenosine triphosphate (ATP) is achieved via both aerobic and anaerobic respiration and fermentation of arginine or pyruvate (Davies *et al.*, 1989; Eschbach *et al.*, 2004; Palleroni, 1992; Vander Wauven *et al.*, 1984; Zannoni, 1989). Respiration requires an external electron acceptor for the complete oxidation of organic compounds. Hereby electrons are transferred to the external electron acceptor via an electron transport chain, which translocates protons in the periplasm and thereby generates an electrochemical gradient (proton motive force, PMF) across the cytoplasmic membrane essential for ATP production. Without available electron acceptors, fermentation of organic substances via substrate-level phosphorylation can be used for ATP synthesis.

1.2.1 Aerobic respiration in *Pseudomonas aeruginosa*

Aerobic respiration requires oxygen as terminal electron acceptor for the oxidation of various carbon sources. The initial oxidations via substrate-specific dehydrogenases are linked to the reduction of oxygen to water by a set of reactions of electron transfer components. Electron transfer components include quinones, cytochromes and terminal oxidases, which contain hemes, [4Fe-4S]-clusters and metals, *e. g.* iron and copper, as redox center, reviewed in (Williams *et al.*, 2007).

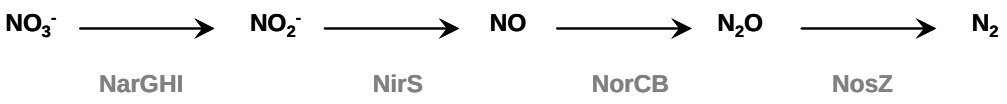
P. aeruginosa has an efficient and very complex aerobic respiration system consisting of genes encoding multiple primary dehydrogenases, ubiquinones, *b*- and *c*-type cytochromes and at least five terminal oxidases (Stover *et al.*, 2000; Williams *et al.*, 2007).

1.2.2 Anaerobic respiration and fermentation in *Pseudomonas aeruginosa*

Anaerobic conditions were shown to be important for biofilm formation and infections of the CF lung and thus will be explained in more detail below (Hassett *et al.*, 2002; Worlitzsch *et al.*, 2002; Yoon *et al.*, 2002). *P. aeruginosa* is capable to grow under anaerobic conditions by nitrate or nitrite respiration and by arginine fermentation (Vander Wauven *et al.*, 1984; Zumft, 1997). Anaerobic survival without growth is possible via pyruvate fermentation (Eschbach *et al.*, 2004).

1.2.2.1 Denitrification in *Pseudomonas aeruginosa*

Under anaerobic conditions, denitrification is the most efficient energy generation system of *P. aeruginosa*. Instead of oxygen, nitrate or nitrite is used as terminal electron acceptor for the oxidation of carbon compounds. Denitrification describes the assemblage of nitrate respiration, nitrite respiration combined with NO reduction and N_2O respiration catalyzed by the nitrate reductase NarGHI, the nitrite reductase NirS, the nitric oxide reductase NorCB and the nitrous oxide reductase NosZ (Zumft, 1997).



Reduction of nitrate to nitrite is catalyzed by the dissimilatory nitrate reductase NarGHI consisting of three subunits (Philippot and Hojberg, 1999; Zumft, 1997). NarI, a membrane-bound cytochrome *b*, transfers the electrons across the membrane via two *b*-hemes from the quinone pool to the cytoplasmic subunit NarH, which in turn transports the electrons via its [4Fe-4S]-clusters to the molybdenum cofactor of NarG, where nitrate is reduced to nitrite. NarGHI is encoded by the *narK$_1$K$_2$GHJI* operon (Schreiber *et al.*, 2007). NarK$_1$K$_2$ are two transmembrane proteins of which only NarK$_2$ seems to be essential for nitrate/nitrite transport (Sharma *et al.*, 2006). NarJ is not part of the nitrate reductase, but is involved in assembly of the enzyme (Zumft, 1997). Expression of the *narK$_1$K$_2$GHJI* operon depends on the oxygen sensing regulator Anr, the N-oxide sensing regulator Dnr and the nitrate response two-component system NarX-NarL, respectively (Schreiber *et al.*, 2007).

P. aeruginosa harbors a second dissimilatory nitrate reductase NapABC consisting of three subunits (Philippot and Hojberg, 1999; Zumft, 1997). NapC, a membrane-bound cytochrome *c*, transports the electrons from a quinone pool to NapB, a *c*-type cytochrome located in the periplasm. NapB transfers the electrons to NapA consisting of a [4Fe-4S]-cluster and a molybdenum center. In contrast to NarGHI, NapABC is not involved in translocation of protons and therefore not used for energy generation. However, it is capable of reducing nitrate under aerobic and anaerobic conditions (Davies *et al.*, 1989). It may function as terminal oxidase sustaining the redox balance of the cell, which is important during transitions from aerobic to anaerobic conditions or aerobic denitrification (Philippot and Hojberg, 1999). NapABC is encoded by the *napEFDABC* operon, which expression was shown to be independent of oxygen and nitrate or nitrite (Schreiber *et al.*, 2007). NapE is proposed to mediate interactions between NapC and a quinol oxidase, NapF is an iron-sulfur protein and NapD may function in maturation of NapA (Schreiber *et al.*, 2007).

Conversion of nitrite to nitric oxide is catalyzed by the nitrite reductase NirS, a cytochrome *cd$_1$* reductase located in the periplasm. It consists of two identical subunits, each carrying a heme *c* and heme *d$_1$* cofactor. The *nirS* gene is organized in the large *nirSMCFDLGHJEN* gene cluster, which is induced by the N-oxide sensing regulator Dnr (Arai *et al.*, 1995a). The *nirS* gene is followed by two *c*-type cytochromes encoded by *nirM*, a cytochrome *c$_{551}$*, and *nirC*, a periplasmic

cytochrome *c*. Both act as electron donors of NirS (Arai *et al.*, 1990; Hasegawa *et al.*, 2001). Moreover, the blue copper protein azurin can act as electron carrier of NirS (Arvidsson *et al.*, 1989). Encoded proteins of the *nirFDLGHJE* genes are required for the biosynthesis of heme d_1 (Kawasaki *et al.*, 1995). The terminal gene *nirN* encodes another periplasmic cytochrome *c* with currently unknown function (Hasegawa *et al.*, 2001).

Reduction of highly toxic nitric oxide to nitrous oxide is catalyzed by the membrane-bound nitric oxide reductase NorCB. The genes encoding the nitric oxide reductase (*norCB*) are located upstream of *nirS* and encode a cytochrome *c* (*norC*) and a cytochrome *b* (*norB*) (Arai *et al.*, 1995b). The *norCB* genes are clustered with *norD* encoding a probable denitrification protein essential for growth on nitrate under anaerobic conditions (Arai *et al.*, 1995b). Transcription of the *norCBD* operon depends on the transcriptional regulator Dnr (Arai *et al.*, 1995b).

Conversion of nitrous oxide to dinitrogen is catalyzed by the nitrous oxide reductase NosZ located in the periplasm. NosZ is a homodimer containing two copper centers in each subunit. The *nosZ* gene is organized in the *nosRZDFYL* gene cluster suggested to be transcribed as an operon (Arai *et al.*, 2003). In *Pseudomonas stutzeri*, the *nosR* gene encodes a membrane-bound regulator essential for NosZ activity, the *nosDFY* gene products are proposed to be involved in processing and insertion of copper into NosZ and *nosL* encodes a putative outer membrane lipoprotein (Zumft, 1997). Expression of the *nosRZDFYL* operon depends, similar to the *nir* and *nor* genes, on the N-oxide regulator Dnr (Arai *et al.*, 2003).

Fig. 1.2 shows a schematic representation of the complete denitrification pathway in *P. aeruginosa*.

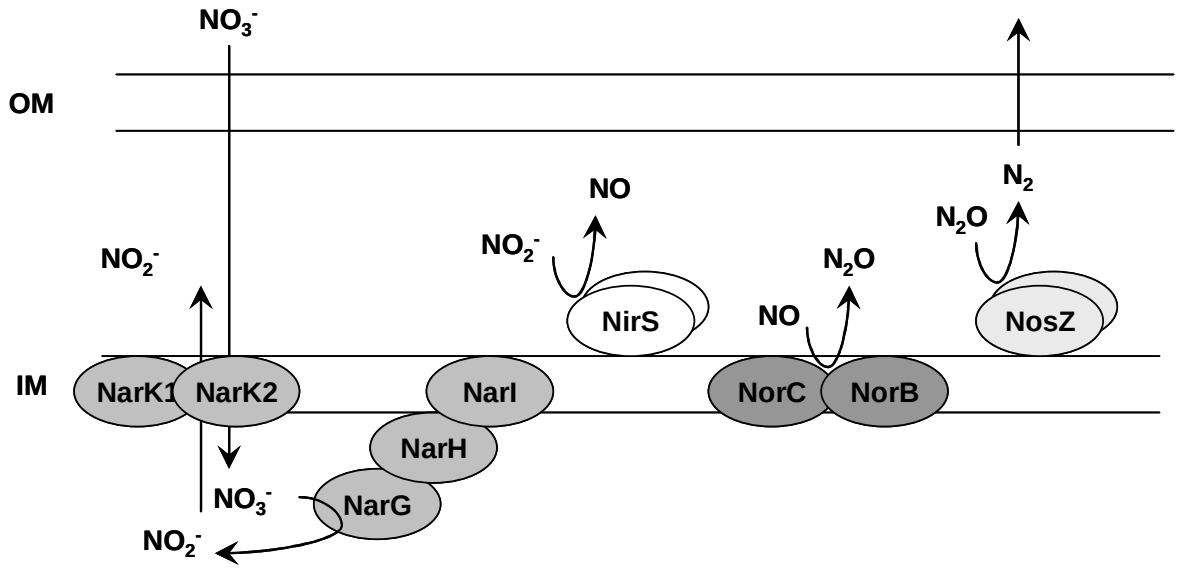


Figure 1.2: Schematic representation of the denitrification pathway in *P. aeruginosa*.
Nitrate is transported via $NarK_2$ into the cell, where NarGHI catalyzes the first step in denitrification, the reduction of nitrate to nitrite. The periplasmic NirS reductase reduces nitrite to nitric oxide, followed by the conversion to nitrous oxide via the NorCB reductase. At least the NosZ reductase converts nitrous oxide to molecular dinitrogen.

1.2.2.2 Fermentation in *Pseudomonas aeruginosa*

1.2.2.2.1 Arginine fermentation

In the absence of oxygen and exogenous electron acceptors *P. aeruginosa* is able to grow by arginine fermentation. Arginine degradation via the arginine deiminase (ADI) pathway permits low energy generation in form of ATP via substrate-level phosphorylation. Conversion of arginine into ornithine and carbamoylphosphate and finally CO_2 and NH_3 with the concomitant generation of ATP is catalyzed by the arginine deiminase ArcA, the catabolic ornithine carbamoyltransferase ArcB and the carbamate kinase ArcC (Vander Wauven *et al.*, 1984), see fig. 1.3. ArcD, an arginine/ornithine antiporter located in cytoplasmic membrane, is additionally required (Verhoogt *et al.*, 1992).

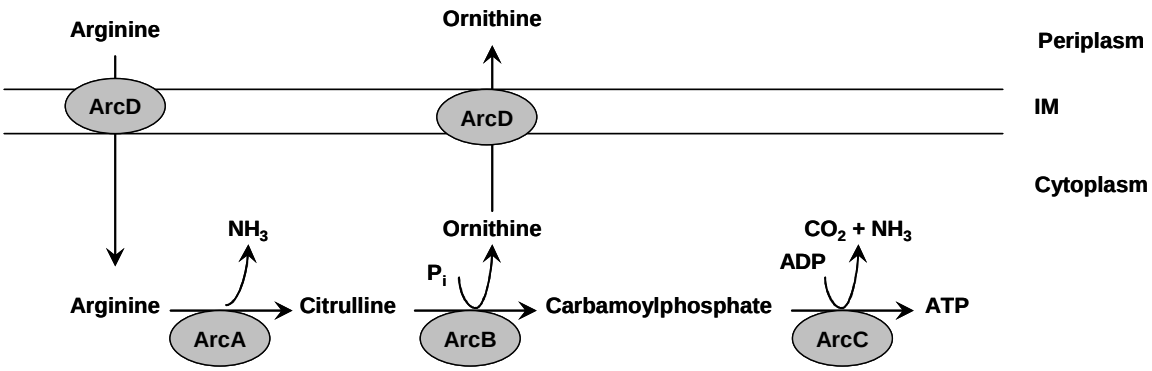


Figure 1.3: Schematic representation of the arginine fermentation pathway in *P. aeruginosa*.
The transport of arginine into the cytoplasm via ArcD is coupled with the export of ornithine. The conversion of arginine via the arginine deiminase (ADI) pathway is catalyzed by ArcA, ArcB and ArcC and provides one molecule of ATP per one molecule arginine.

The genes encoding the enzymes essential for arginine fermentation are organized in the *arcDABC* operon (Vander Wauven *et al.*, 1984). Expression of the *arcDABC* operon requires oxygen limitation and depends on Anr (Galimand *et al.*, 1991; Gamper *et al.*, 1991). Moreover, transcription of *arcDABC* is stimulated in the presence of exogenous arginine by the arginine responsive regulator ArgR acting as transcriptional regulator by binding to a conserved sequence (Lu *et al.*, 1999). The ArgR binding site is located upstream of the Anr box in the *arcDABC* promoter proposing an interaction of these two regulators (Lu *et al.*, 1999).
Interestingly, enzyme activity of ArcB was found decreased in the presence of nitrate implying an interconnection between denitrification and arginine fermentation (Mercenier *et al.*, 1980).

1.2.2.2.2 Pyruvate fermentation

In the absence of oxygen and alternative electron acceptors *P. aeruginosa* can also perform pyruvate fermentation. Pyruvate is metabolized into the end products acetate, lactate and succinate, see fig. 1.4 (Eschbach *et al.*, 2004). However, pyruvate fermentation supports long-term survival of *P. aeruginosa*, but no growth.

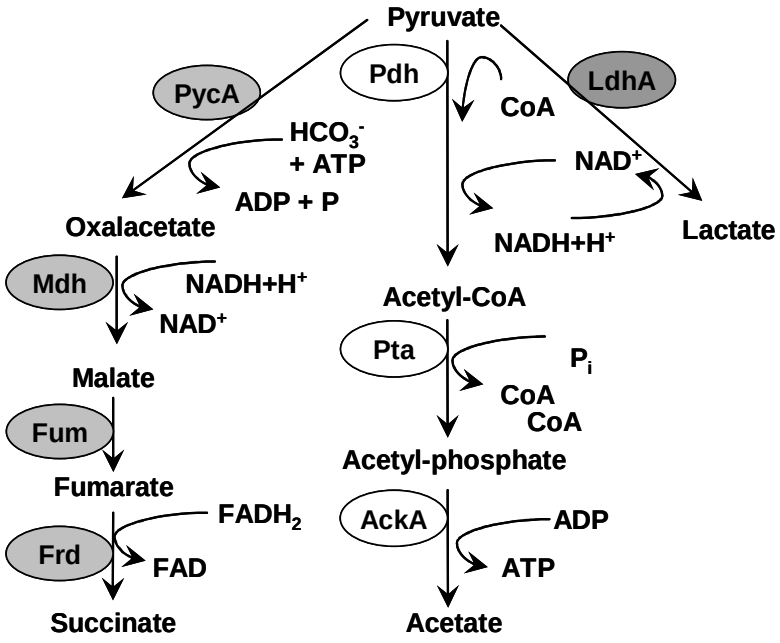


Figure 1.4: Schematic representation of pyruvate fermentation in *P. aeruginosa*, modified from (Eschbach *et al.*, 2004).

In *P. aeruginosa* pyruvate fermentation includes a set of reactions for the conversion of pyruvate. On one hand, PycA (pyruvate carboxylase), Mdh (malate dehydrogenase), Fum (fumarase) and Frd (fumarate reductase) are used for the conversion of pyruvate into succinate. On the other hand pyruvate is metabolized by Pdh (pyruvate dehydrogenase), Pta (phosphotransacetylase) and AckA (acetate kinase) resulting in the production of acetate. Additionally pyruvate is degraded into lactate by the LdhA (lactate dehydrogenase).

It was shown, that expression the *ackA-pta* operon encoding an acateate kinase and a phosphotransactylase is under control of the anaerobic regulator Anr and the DNA bending protein IHF (Eschbach *et al.*, 2004).

1.2.3 Regulation of anaerobic metabolism in *P. aeruginosa*

Anaerobic growth of *P. aeruginosa* via arginine fermentation and denitrification generally requires the oxygen sensing regulator Anr (anaerobic regulation of arginine deiminase and nitrate reduction) (Galimand *et al.*, 1991; Ye *et al.*, 1995). Moreover the N-oxide regulator Dnr (dissimilatory nitrate respiration regulator) and the nitrate responsive two-component system NarX-NarL are essential for anaerobic denitrifying growth (Schreiber *et al.*, 2007).

The complete anaerobic regulatory network required for anaerobic denitrifying growth was recently published by our group (Schreiber *et al.*, 2007). The major

regulator Anr is on the top of the regulatory cascade and induces expression of the denitrification genes directly by inducing expression of the *narK$_1$K$_2$GHJI* operon and indirectly via the activation of Dnr and NarX-NarL, see fig. 1.5. Dnr activates expression of the reductases essential for conversion of nitrite to dinitrogen (*nir*, *nor* and *nos*) and enhances transcription of the *nar* genes. NarL induces transcription of the nitrate reductase operon *narK$_1$K$_2$GHJI* and additionally *nirQ*, encoding a putative ATP-binding protein found to be essential for post-transcriptional activation of the NirS and NorCB reductases in *P. aeruginosa* and *P. stutzeri*, see fig. 1.5 (Arai *et al.*, 1994; Jungst and Zumft, 1992).

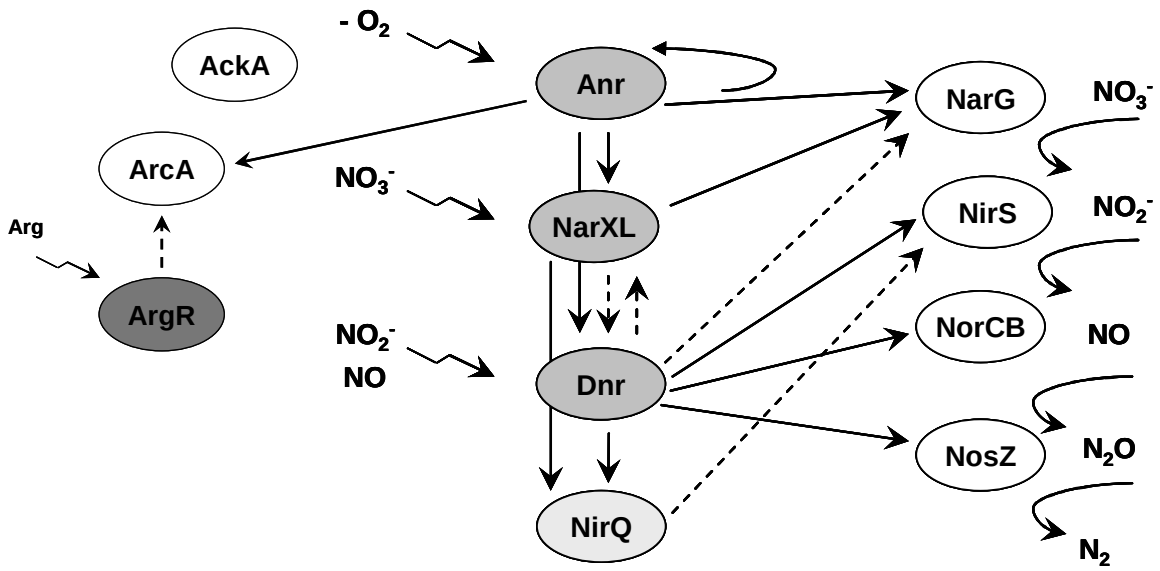


Figure 1.5: Schematic representation of the anaerobic regulatory network of *P. aeruginosa*, modified from (Schreiber *et al.*, 2007).

Low oxygen conditions lead to the activation of Anr, which induces denitrification by activating expression of the other two transcriptional regulators Dnr and NarL, the nitrate reductase NarGHI and the own one. Dnr activates transcription of the *nir*, *nor* and *nos* genes and enhances expression of the *nar* operon. Active NarL was found to induce the *nar* genes and *nirQ*. It was also shown, that NarL and Dnr enhance mutual expression (Schreiber *et al.*, 2007). Additionally, active Anr activates arginine and pyruvate fermentation by inducing the *arcDABC* and *ackA-pta* operons. Filled arrows represented main activation via the corresponding regulator, interrupted arrows indicated enhanced expression.

1.2.3.1 The oxygen sensing regulator Anr

Anr is a one-component regulatory system acting as redox sensor and transcriptional regulator. The *P. aeruginosa* Anr shows 51 % amino acid sequence identity to the *Escherichia coli* homologue Fnr (fumarate and nitrate reduction) (Sawers, 1991). Both belong to the Crp-Fnr family of transcriptional regulators and carry four N-terminal cysteine residues essential for assembly of a [4Fe-4S]-cluster sensing intracellular oxygen (Körner *et al.*, 2003; Sawers, 1991). In *E. coli*, Fnr is inactivated via its oxygen labeled iron-sulfur cluster upon prolonged oxygen exposure. Below a certain threshold concentration of dissolved oxygen of about 10 µM, the [4Fe-4S]-cluster is reassembled. Active Fnr forms a dimer regulating transcription of target genes by binding to a conserved DNA sequence located up to 40 bp upstream of the transcriptional start known as Fnr box (TTGATNNNNATCAA) (Spiro and Guest, 1990). Anr binds to a DNA binding site similar to the Fnr box (Galimand *et al.*, 1991; Winteler and Haas, 1996).

1.2.3.2 The N-oxide sensing regulator Dnr

Anr controls the expression of genes required for denitrification by inducing *dnr*, which encodes a second transcriptional Crp-Fnr regulator (Arai *et al.*, 1995a; Arai *et al.*, 1997). Dnr displays an amino acid sequence similarity of 25 % to Anr. In contrast to the Anr and Fnr proteins, Dnr lacks in N-terminal cysteine residues required for oxygen sensing in the cell via a [4Fe-4S]-cluster, but was shown to be involved in sensing N-oxides (Arai *et al.*, 1995a; Hasegawa *et al.*, 1998). Recently, the structure of Dnr was published showing that the sensory domain is significantly rotated compared to the orientation in other Crp-Fnr family regulators (Giardina *et al.*, 2008; Giardina *et al.*, 2009). Dnr binds to a consensus sequence indistinguishable to the Anr box. However, it specifically regulates expression of the denitrification genes *nirS*, *norCB*, *nosZ* and *nirQ* essential for the conversion of nitrite to dinitrogen (Arai *et al.*, 1999; Arai *et al.*, 2003; Hasegawa *et al.*, 1998; Schreiber *et al.*, 2007; Trunk, 2005). Additionally, genes regulated by both, Anr and Dnr, via one Anr box were described, *e. g. hemA*, *hemF*, *hemN* and *narK$_1$K$_2$GHJI* (Krieger *et al.*, 2002; Rompf *et al.*, 1998; Schreiber *et al.*, 2007).

1.2.3.3 The nitrate sensing two-component system NarX-NarL

Anr also controls the expression of the *narXL* operon encoding a nitrate responsive two-component system (Schreiber *et al.*, 2007). The two-component system consists of the sensor kinase NarX and the cognate response regulator NarL. The *P. aeruginosa* NarX shows 38 % and 34 % sequence identity to NarX from *P. stutzeri* and *E. coli* and NarL 63 % and 58 % to NarL from *P. stutzeri* and *E. coli*, respectively (Härtig *et al.*, 1999). In presence of nitrate, the membrane-bound sensor NarX is activated by auto-phosphorylation and in turn phosphorylates NarL. *P. aeruginosa* NarL acts as transcriptional regulator by binding to a specific DNA sequence in the promoter region of target genes similar to the *E. coli* NarL binding site ($TA^{C}/_{T}N^{C}/_{A}T$) (Krieger *et al.*, 2002; Schreiber *et al.*, 2007; Tyson *et al.*, 1993).

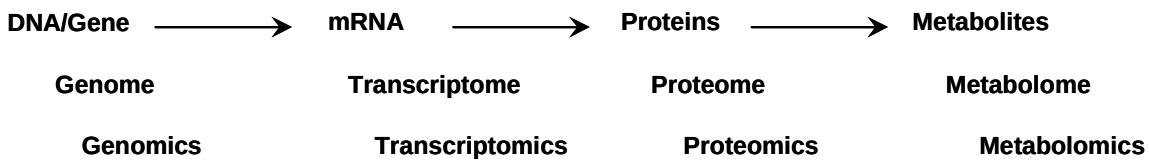
Recently, a comprehensive microarray analysis of the *E. coli* NarX-NarL regulon identified NarL-dependent expression of more than 90 operons and demonstrated the involvement in coordination of the anaerobic energy metabolism by inducing nitrate respiration and repressing less efficient modes of energy metabolism including fermentation processes (Constantinidou *et al.*, 2006). In contrast to *E. coli*, only a few genes were shown to be targets of NarL activation in Pseudomonads (Härtig *et al.*, 1999; Krieger *et al.*, 2002; Schreiber *et al.*, 2007; Vollack *et al.*, 1999).

1.3 SYSTEMS BIOLOGY

Systems biology is a comprehensive quantitative analysis of the manner in which all components of a biological system functionally interact over time (Aderem, 2005). It involves high-throughput “wet” experiments, technological development, theory and computational modeling (Kell, 2006). The ultimate goal of systems biology is the understanding and prediction of complex biological systems.

1.3.1 Experimental “omics” approaches

For the collection of quantitative high-throughput data the experimental approach using “omics” techniques (genomics, transcriptomics, proteomics and metabolomics) is essential.



Genomics is the analysis of the entire genome of a biological system and is based on DNA sequence determination and genetic mapping. It allows the identification of genes and its predicted or annotated functions.

Transcriptomics describes the analysis of the transcriptome, the complete set of a systems mRNA encoded by the genome, via DNA microarrays. Combined with genomics, it provides a method for regulatory network determination.

Proteomics means the identification of a systems proteome, the complete set of proteins encoded by the genome, via two-dimensional (2D) gel electrophoresis and mass spectrometry (MS), *e. g.* matrix assisted laser desorption/ionization - time of flight (MALDI-TOF) as well as electrospray ionization- (ESI) and liquid chromatography- (LC) MS/MS.

Metabolomics is the study of a systems metabolome, the whole set of metabolites, under a given set of conditions (Goodacre *et al.*, 2004). The metabolome represents the end products of a regulatory process and therefore shows the final response of a biological system. Techniques used in metabolomics are mainly nuclear magnetic resonance (NMR) spectroscopy and chromatographic mass spectrometry.

Whereas the genome of an organism is stable, the latter three “omics” (transcriptomics, proteomics and metabolomics) change in response to environmental conditions and focuse on dynamic functions and interactions. To date they are limited by a number of cellular processes, *e. g*. mRNA stability, translational control, protein modification and degradation. Nevertheless, computational data integration and modeling are required for the reconstruction of biological systems *in silico*.

1.3.1.1 Metabolomics

Metabolomics is a relatively new technique of the “omics” sciences becoming more and more important. Most of the literature available was published in the last years describing bacterial, plant, animal as well as human metabolome analysis.

The methods used for analysis are mostly NMR and MS techniques including GC- (gas chromatography) and LC-MS, reviewed in (Rochfort, 2005).

NMR analysis is a useful method for determination of the metabolome *in vivo* and thus is often applied in clinical studies testing human blood plasma or urine. However, it is limited to a small number of detectable metabolites (Khoo and Al-Rubeai, 2007). Nevertheless, NMR metabolomics was successfully applied to study the differences of *P. aeruginosa* cells grown planktonically and as biofilms (Gjersing *et al.*, 2007). Moreover, NMR analysis of different *Bacillus cereus* strains clearly showed the potential this technique for discrimination of pathogenic and non-pathogenic bacteria (Bundy *et al.*, 2005).

In contrast to NMR, chromatographic techniques such as GC-MS, LC-MS and capillary electrophoresis (CE) are capable to detect more than hundreds of metabolites, but need sample preparation prior analysis (Khoo and Al-Rubeai, 2007). In *Corynebacterium glutamicum*, GC-MS analysis revealed the simultaneous quantitative determination of more than 300 metabolites and showed that the method can be used for both, characterization of different growth conditions or stages and characterization of genetic changes (Strelkov *et al.*, 2004). Further investigations of the *E. coli* and *P. aeruginosa* metabolome demonstrated the ability of CE for metabolic differentiation of bacteria adapted to various antibiotics (Gao *et al.*, 2007).

1.4 AIMS OF THE STUDY

Anaerobic growth and persistence are major prerequisites for a successful infection by the opportunistic pathogen *P. aeruginosa*. The underlying regulatory network was the major target of this thesis. First, open questions concerning the three major anaerobic regulatory systems of *P. aeruginosa*, Anr, Dnr and NarXL, had to be approached experimentally. Since only a few genes are known to be regulated by the two-component nitrate response regulator NarL, a transcriptomic approach should help to identify new members of the NarL regulon. NarL mediated repression of the arginine fermentation genes *arcDABC*, which was identified by proteome analysis in our laboratory before, should be confirmed using Northern blot analysis and promoter *lacZ* reporter gene assay. Both redox response regulators, Anr and Dnr, control expression of target genes by binding to an indistinguishable binding site in the promoter. A bioinformatics analysis previously performed in our laboratory failed to detect regulator specific promoter sequences. Thus, an Anr- (*arcD*) and Dnr-dependent (*nirS*) promoter should be investigated for elements mediating Anr and Dnr specific gene regulation using promoter *lacZ* reporter gene studies.

In the second part of this thesis, the correlation of transcriptional and metabolic regulation was of interest. A reproducible method for the extraction and measurement of *P. aeruginosa* metabolites using a GC-MS based technique had to be established in cooperation with the group of Professor Schomburg. Next, metabolome in combination with transcriptome analyses should be applied to study the correlation of both regulatory networks. The adaptation during the shift from exponential to stationary growth phase should serve as first example offering new insights into the cellular response to changing growth conditions on both transcriptional and metabolic level.

2 MATERIALS AND METHODS

2.1 INSTRUMENTS AND CHEMICALS

2.1.1 Instruments

Agarose Gel Documentation	ChemiDoc	Bio-Rad
Agarose Gel Electrophoresis	Agagel Mini	Biometra
Autoclave	EL 3850	Syntec
Blotting	Vacu-Blot System with pump MP86	Biometra
Centrifuges	Biofuge® fresco, Megafuge 1.0R	Heraeus
	Minispin®	Eppendorf
Clean bench	Hera Safe	Heraeus
Concentrator	Concentrator 5301	Eppendorf
DNA Sequencing	ABI PRISM™ 310 Genetic Analyser	Applied Biosystems
Electroporation	Gene Pulser II™	Bio-Rad
GC-MS system	Finnigan Trace GC 2000/AS 2000	Thermo Electron
pH Determination	pH 211 Microprocessor pH Meter	Hanna Instruments
Pipettes	Reference, Research	Eppendorf
PAGE	Mini Protean II	Bio-Rad
Scales	Model 572, Model ALJ 160-4NM	Kern
Shaker	Bench Top Shaker, Aquatron	Infors AG HT
Spectrophotometer	NanoDrop ND-1000	Peqlab
	Ultrospec 1100 pro	Amersham
Thermocycler	Tgradient, Tpersonal	Biometra
Thermomixer	Thermomixer compact	Eppendorf
Vortex	Vortex-Genie 2	Scientific Industries
Water Purification	Synthesis A10	Millipore

2.1.2 Chemicals and Materials

Chemicals and enzymes

Blocking reagent	Roche
GeneChip® labeling reagent	Affymetrix
NeutrAvidin™	Molecular Probes
Restriction enzymes, T4 DNA ligase	MBI-Fermentas
Restriction enzymes, CDP star, SAP	New England Biolabs
RNase-Free DNase I	GE Healthcare
Sequencing reagents	Applied Biosystems
SUPERase*In RNase inhibitor	Ambion
Taq DNA polymerase, One-for-all-buffer	Amersham

SuperScript™ II reverse transcriptase, Random primers, dNTPs	Invitrogen
SYBR® Gold Nucleic Acid Gel Stain	Molecular Probes
Terminal Deoxynucleotide Transferase	Promega
Kits	
DIG-RNA Labeling Kit	Roche
QIAquick Gel Extraction Kit, PCR Purification Kit, RNAeasy mini kit	QIAgen
QuikChange™ Site-Directed Mutagenesis Kit	Stratagene
Materials	
Oligonucleotide primers	Metabion
P. aeruginosa GeneChip®	Affymetrix
Sterile filter Filtropur S 0.2 µm	Sarstedt
Molecular weight standards for agarose and polyacrylamide gels	
DIG-labeled RNA Molecular Weight Marker II	Roche
GeneRuler™ DNA Ladder, MassRuler™ DNA Ladder	MBI Fermentas
Hyper Ladder V ™	Bioline

Chemicals and reagents not specifically listed here were purchased from Difco, Fluka, GE Healthcare, Merck, Riedel-de-Häen, Roth, and Sigma-Aldrich.

2.2 STRAINS, PLASMIDS AND OLIGONUCLEOTIDE PRIMERS

2.2.1 Strains

Table 2.1: Strains used in this study.

Bacterial strain	Genotype or phenotype	Reference
P. aeruginosa		
PAO1	wild type	(Dunn and Holloway, 1971)
PAO9104	*narL::cat*, Cm^r	(Krieger *et al.*, 2002)
PAO6261	PAO1 Δ*anr*	(Ye *et al.*, 1995)
BB71	PAO1 Δ*dnr*	(Boes *et al.*, 2006)
BB21	PAO1 *attB*::(P_{arcD}-*lacZ*), Tc^r	This work
BB22	PAO1 *attB*::(P_{nirS}-*lacZ*), Tc^r	This work
BB25	PAO6261 *attB*::(P_{arcD}-*lacZ*), Tc^r	This work
BB26	PAO6261 *attB*::(P_{nirS}-*lacZ*), Tc^r	This work
BB36	PAO1 *attB*::($P_{arcD\text{-}ds\text{-}ex}$-*lacZ*), Tc^r	This work
BB37	PAO1 *attB*::($P_{arcD\text{-}us\text{-}ex}$-*lacZ*), Tc^r	This work
BB38	PAO1 *attB*::($P_{nirS\text{-}ds\text{-}ex}$-*lacZ*), Tc^r	This work
BB39	PAO1 *attB*::($P_{nirS\text{-}us\text{-}ex}$-*lacZ*), Tc^r	This work
BB43	PAO1 *attB*::(P_{arcD}-*lacZ*)	This work
BB45	PAO9104 *attB*::(P_{arcD}-*lacZ*)	This work

BB46	PAO1 *attB*::($P_{arcD\Delta NarL}$-*lacZ*)	This work
BB63	PAO6261 *attB*::($P_{arcD\text{-}us\text{-}ex}$-*lacZ*), Tc^r	This work
BB64	PAO6261 *attB*::($P_{nirS\text{-}ds\text{-}ex}$-*lacZ*), Tc^r	This work
BB72	BB71 *attB*::(P_{arcD}-*lacZ*), Tc^r	This work
BB73	BB71 *attB*::(P_{nirS}-*lacZ),* Tc^r	This work
BB80	BB71 *attB*::($P_{arcD\text{-}us\text{-}ex}$-*lacZ*), Tc^r	This work
BB81	BB71 *attB*::($P_{nirS\text{-}ds\text{-}ex}$-*lacZ*), Tc^r	This work
BB82	PAO6261 *attB*::($P_{arcD\text{-}ds\text{-}ex}$-*lacZ*), Tc^r	This work
BB83	BB71 *attB*::($P_{arcD\text{-}ds\text{-}ex}$-*lacZ*), Tc^r	This work
BB84	PAO6261 *attB*::($P_{nirS\text{-}us\text{-}ex}$-*lacZ*), Tc^r	This work
BB85	BB71 *attB*::($P_{nirS\text{-}us\text{-}ex}$-*lacZ*), Tc^r	This work
E. coli		
DH10B	F^- *mcrA* Δ(*mrr-hsd*RMS-*mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX*74 *deoR recA*1 *endA*1 *araD*139 Δ(*ara*, *leu*) 7697 *galU galK* λ^- *rpsL nupG*	Invitrogen
S17 λ-*pir*	*pro thi hsdR*$^+$ Tp^r Sm^r; chromosome::RP4-2 Tc::Mu-Km::Tn7/λ*pir*	(Simon, 1983)
SM10	*thi*-1 *thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r	(de Lorenzo and Timmis, 1994)

2.2.2 Plasmids

Table 2.2: Plasmids used in this study.

Plasmid	Genotype/phenotype	Reference
Mini-CTX-*lacZ*	promoterless *lacZ* gene, Tc^r	(Becher and Schweizer, 2000)
pFLP2	source of FLP recombinase, Ap^r	(Hoang *et al.*, 1998)
pDH11	mini-CTX-*lacZ* containing a 1005 bp *Pst*I-*Bam*HI fragment encoding the *arcD* promoter, Tc^r	Dana Heldt
pDH12	mini-CTX-*lacZ* containing a 648 bp *Pst*I-*Bam*HI fragment encoding the *nirS* promoter, Tc^r	Dana Heldt
pBB16	pDH11 with exchanged downstream (ds) region of pDH12, Tc^r	This work
pBB17	pDH12 with exchanged ds region of pDH11, Tc^r	This work
pBB18	pDH11 with exchanged upstream (us) region of pDH12, Tc^r	This work
pBB19	pDH12 with exchanged us region of pDH11, Tc^r	This work
pBB20	pDH11 with mutated NarL binding site, Tc^r	This work

2.2.3 Oligonucleotide primers

Table 2.3: Oligonucleotide primers used for PCR.

Recognition sequences for restriction enzymes are underlined and mutated bases are printed fat. Binding sites for the corresponding transcriptional regulators Anr (TTGACGTGGATCAG), Dnr (TTGATTCCGGTCAA) and NarL (TACTCAA → CATTCAA) are marked in grey.

Primer designation	Primer sequence (5' - 3')	Construct
ArcD_for_A	AACTGCAGGCTGCCGTGGCTCATGAT	P_{arcD}
ArcD_rev_B	CGGGATCCTTTGCGGGAGGGAGAAGA	P_{arcD}
NirS_for_A	AACTGCAGATCAGATGGCGGCCGAG	P_{nirS}
NirS_rev_B	CGGGATCCTCTGCTTGGCCTCGTTGA	P_{nirS}
oBB36_for	TTGATTCCGGTCAAGCAAGGGTAAA	$P_{nirS\text{-}ds}$
oBB37_rev	TTGACCGGAATCAAGATTGCGTTGC	$P_{nirS\text{-}us}$
oBB40_for	TTGACGTGGATCAGCATTCAACAAT	$P_{arcD\text{-}ds}$
oBB41_rev	CTGATCCACGTCAATAGCTTCCTAC	$P_{arcD\text{-}us}$
oBB42_arc-nar_rev	AATAGCTTCC**CA**T**T**CAAAGTAATTAGAT	$P_{arcD\Delta NarL}$
oBB43_arc-nar_for	ATCTAATTACTTTGA**AT****G**GGAAGCTATT	$P_{arcD\Delta NarL}$
oKS44_CTX_rev	GCGCATCGTAACCGTGCATC	
oKS47_CTX_for	GCGCTTTTGAAGCTGATGTG	

2.3 GROWTH MEDIA AND MEDIA ADDITIVES

2.3.1 Growth media

Luria Bertani (LB) medium was used as standard medium for growth of *E. coli* and *P. aeruginosa* (Sambrook and Russell, 2001). Modified AB minimal medium was applied as minimal medium for *P. aeruginosa* (Clark, 1967; Schreiber *et al.*, 2006). For solid media, 1.5 % (w/v) agar was added before sterilization.

LB medium	tryptone	10 g
	yeast extract	5 g
	NaCl	5 g
	H_2O_{deion}	ad 1 l
AB minimal medium	A10 salt solution	100 ml
	glucose	20 mM
	trace metal solution	1 ml
	$MgCl_2$	1 mM
	$FeSO_4$	25 µM
	$CaCl_2$	100 µM
	H_2O_{deion}	ad 1 l

A10 salt solution (pH 7.0)	$(NH_4)_2SO_4$	150 mM
	Na_2HPO_4	335 mM
	KH_2PO_4	220 mM
	NaCl	500 mM
	dissolved in H_2O_{deion}	
Trace metal solution	$CaSO_4$ x 2 H_2O	200 mg
	$FeSO_4$ x 7 H_20	200 mg
	$MnSO_4$ x H_2O	20 mg
	$CuSO_4$ x 5 H_2O	20 mg
	$ZnSO_4$ x 7 H_2O	20 mg
	$CoSO_4$ x 7 H_2O	10 mg
	Na_2MoO_4 x H_2O	10 mg
	H_3BO_4	5 mg
	H_2O_{deion}	ad 1 l

2.3.2 Media additives

Table 2.4: Media additives

Antibiotics and other sensitive additives were prepared as concentrated stock solutions, sterilized by filtration and added after autoclaving to the chilled medium.

Substances	**Solute**	**Stock solution**	**Final concentration**	
Antibiotics			***E. coli***	***P. aeruginosa***
carbenicillin	H_2O_{deion}	100 mg ml^{-1}	100 µg ml^{-1}	500 µg ml^{-1}
chloramphenicol	EtOH 70 % (v/v)	100 mg ml^{-1}	30 µg ml^{-1}	500 µg ml^{-1}
gentamicin	H_2O_{deion}	30 mg ml^{-1}	10 µg ml^{-1}	200 µg ml^{-1}
tetracycline	EtOH 70 % (v/v)	10 mg ml^{-1}	5 µg ml^{-1}	100 µg ml^{-1}
Other additives				
glucose	H_2O_{deion}	1 M	-	20 mM
$CaCl_2$	H_2O_{deion}	1 M	-	100 µM
KNO_3	H_2O_{deion}	1 M	-	50 mM
$MgCl_2$	H_2O_{deion}	1 M	-	1 mM
$NaNO_2$	H_2O_{deion}	1 M	-	10 mM
ONPG	H_2O_{deion}	4 mg ml^{-1}	-	800 µg ml^{-1}
ribitol	H_2O_{deion}	200 µg l^{-1}	-	1 µg ml^{-1}
sucrose	H_2O_{deion}	50 % (w/v)	-	5 % (w/v)

2.4 MICROBIOLOGICAL TECHNIQUES

2.4.1 Sterilization

Unless noted otherwise, all media and solutions were vapor sterilized at 121 °C and 1 bar positive pressure for 20 min. Temperature sensitive solutions were sterilized by filtration.

2.4.2 Cultivation of bacteria

Bacteria were plated directly from a glycerol stock onto a medium agar plate and incubated overnight at 37 °C. For liquid overnight cultures, 5 ml medium in test tubes were inoculated using a single colony from an agar plate and shaken aerobically overnight at 37 °C and 200 rpm. For aerobic liquid main cultures, 60 to 200 ml medium in Erlenmeyer flasks were inoculated 1/10 with the corresponding overnight culture (start OD_{578} ~ 0.05) and were incubated at 37 °C and 200 rpm. For anaerobic liquid cultures 100 to 140 ml medium in sealed serum flasks were inoculated 1/10 with the corresponding overnight culture (start OD_{578} ~ 0.05) and incubated at 37 °C and 100 rpm. Incubation times varied according to the desired optical densities.

2.4.3 Determination of cell density

Cell densities of liquid cultures were determined by measuring the optical density at a wavelength of 578 nm (OD_{578}). An OD_{578} of 1 corresponded to approximately 1 x 10^9 cells per ml. Samples with an OD_{578nm} > 1 were diluted 1/10 in the corresponding medium for measurement.

2.4.4 Storage of bacteria

Bacterial strains were kept on medium agar plates for up to 10 days at 4 °C. For long-term storage of bacteria, glycerol cultures were prepared using an overnight culture with a final concentration of 13 % (w/v) glycerol and stored at - 80 °C.

2.5 MOLECULAR BIOLOGICAL TECHNIQUES

Protocols employed in this study are generally based on the methods described by Sambrook (Sambrook and Russell, 2001). Modifications are described below.

2.5.1 Preparation of DNA/RNA

2.5.1.1 Preparation of genomic DNA

Four ml of an overnight culture were harvested by centrifugation (16,000 x g; 2 min; 4 °C) and suspended in 700 µl of buffer P1 (see 2.5.1.2). An equal volume of phenol-chloroform-isoamylalcohol (PCI, 25:24:1) was added to the suspension. Following centrifugation (16,000 x g; 15 min; 4 °C), 700 µl of the supernatant were added to 700 µl PCI (25:24:1) and centrifuged (16,000 x g; 15 min; 4 °C). After this step was repeated, 600 µl of the supernatant were added to 600 µl chloroform and centrifuged (16,000 x g; 15 min; 4°C). Precipitation of DNA was allowed by adding 60 µl Na-acetate (3 M; pH 5) and 700 µl isopropanol. Following centrifugation (16,000 x g; 15 min; 4 °C), DNA was washed with 500 µl 70 % (v/v) ethanol. After all traces of ethanol had evaporated, DNA was solubilized in 50 µl H_2O_{deion}.

2.5.1.2 Preparation of plasmid DNA (Mini Prep)

Four ml of an overnight culture containing the plasmid of interest were harvested by centrifugation (16,000 x g; 5 min; 4 °C) and cells were suspended in 300 µl of buffer P1. Suspension was carefully mixed with 300 µl of buffer P2 and incubated for 5 min at RT. Then 300 µl of buffer P3 were added and again suspension was carefully mixed and incubated for 5 min at RT. Following centrifugation (16,000 x g; 10 min; 4 °C) 800 µl of the supernatant were added to 560 µl isopropanol. Precipitation of plasmid DNA was allowed to proceed during centrifugation (16,000 x g; 30 min; 4 °C). DNA was washed with 500 µl 70 % (v/v) ethanol. After all traces of ethanol had evaporated, DNA was solubilized in 50 µl H_2O_{deion}.

Buffer P1 (pH 8.0)	glucose	50 mM
	Tris-HCl	25 mM
	EDTA	10 mM
	dissolved in H_2O_{deion}	
Buffer P2	NaOH	200 mM
	SDS	1.0 % (w/v)
	dissolved in H_2O_{deion}	
Buffer P3 (pH 5.3)	Na-acetate	3 M
	dissolved in H_2O_{deion}	

2.5.1.3 Preparation of total RNA

RNA was prepared by a modified hot phenol method (Aiba *et al.*, 1981; von Gabain *et al.*, 1983). 5 - 25 ml of a *P. aeruginosa* culture were immediately mixed with 25 ml ice-cold killing solution supplemented with 250 µl NaN_3 (130 mg ml^{-1}). After centrifugation (4,000 x g; 4 °C; 10 min) cells were shock frozen in liquid nitrogen and stored at - 20 °C. For RNA isolation cells were suspended in 125 µl ice-cold sucrose/Na-acetate and gently mixed with 125 µl SDS/Na-acetate. After incubation for 90 sec at 65 °C the suspension was supplemented with 400 µl hot phenol and incubated for 3 min at 65 °C. After shock freezing in liquid nitrogen and centrifugation (12,000 x g; 10 min; RT) the supernatant was mixed with 400 µl hot phenol and incubated for 3 min at 65 °C. Again suspension was shock frozen and centrifuged (12,000 x g; 10 min; RT). Supernatant was mixed with 200 µl PCI (25:24:1) and centrifuged (12,000 x g; 3 min; RT). This step was repeated two times. The supernatant was then mixed with 200 µl chloroform-isoamylalcohol (24:1) and centrifuged (12,000 x g; 3 min; RT). Resulting supernatant was added to 40 µl Na-acetate (3 M; pH 5.2) and 1 ml 100 % ethanol (v/v) and for precipitation of RNA incubated overnight at - 20 °C. After centrifugation (12,000 x g; 20 min; RT) the precipitate was solubilized in 180 µl storage buffer and 20 µl DNase buffer were added. RNA solution was treated with 0.4 µl RNase free DNase I (10 U $µl^{-1}$) for 30 min at RT and further purified with the RNAeasy mini kit.

Killing solution (pH 7.5)	Tris-HCl	20 mM
	$MgCl_2$	5 mM
	dissolved in H_2O_{deion}	
Sucrose/Na-acetate	sucrose	300 mM
	Na-acetate	10 mM
	dissolved in H_2O_{deion}	
SDS/Na-acetate	SDS	2 % (w/v)
	Na-acetate	10 mM
	dissolved in H_2O_{deion}	
Storage buffer	$NaPO_4$	20 mM
	EDTA	1 mM
	dissolved in H_2O_{deion}	
10 x DNase buffer	Na-acetate	200 mM
	$MgCl_2$	100 mM
	NaCl	100 mM
	dissolved in H_2O_{deion}	

2.5.2 Polymerase chain reaction (PCR)

2.5.2.1 PCR conditions

For amplification of DNA, the polymerase chain reaction (PCR) method was used. After an initial DNA denaturation step for 5 min at 95 °C, a cycle consisting of the three steps including denaturation, primer annealing and elongation was completed 30 times and at last terminated by a final elongation step for 10 min at 72 °C. Denaturation and elongation temperature remained unchanged, whereas annealing temperature depended on oligonucleotide length and G + C content and was furthermore influenced by the insertion of mismatches. Time of the elongation was chosen according to the length of DNA fragments to be amplified according to manufacturer's instructions.

PCR reaction mix	DNA	10 ng	
	10 x PCR buffer	5 µl	
	primer	20 pmol	
	dNTPs	200 µM	
	Taq DNA polymerase	0.5 U	
	H_2O_{deion}	ad 50 µl	
Standard PCR program	95 °C	5 min	
	95 °C	1 min	 30 x
	primer-dependent	1 min	
	72 °C	30 - 90 sec	
	72 °C	5 min	

2.5.2.2 Purification of PCR products

For quality controls of amplified DNA, an aliquot of the PCR reaction mixture was analyzed by agarose gel electrophoresis, see 2.5.8.1. If only the desired PCR product was detected, the entire sample was purified with the QIAquick PCR Purification Kit. If additional PCR products were detected, the entire reaction mixture was first electrophoretically separated and the DNA fragment of interest was excised from the agarose gel and purified using the QIAquick Gel Extraction Kit. All kits were used according to the manufacturer's instructions.

2.5.3 Site-directed mutagenesis of DNA

For the generation of point mutations in diverse promoters, the crossover PCR technique described by Ho or the QuickChange™ Site-directed mutagenesis method was used (Ho *et al.*, 1989). Nucleotide exchanges were verified by DNA sequence determination.

2.5.4 Enzymatic modifications

2.5.4.1 Restriction of DNA

Restriction of DNA (vectors and PCR products) was carried out using restriction endonucleases. Reaction buffers, concentrations of enzymes and DNA as well as incubation temperatures were chosen according to manufacturer's instructions. Digestion was allowed to proceed for 2 - 4 h.

2.5.4.2 Dephosphorylation of vector DNA

In order to avoid re-circularization of digested vector DNA, the 5'-phosphate groups were removed prior ligation by adding shrimp alkaline phosphatase (SAP). Samples were incubated with SAP (1 U μg^{-1} DNA) for 30 min at 37 °C, followed by purification with the PCR purification kit.

2.5.4.3 Ligation of DNA

Ligation of DNA was carried out using T4 DNA ligase (1 - 2 U) in a reaction buffer supplied by the manufacturer. An amount of 25 - 50 ng vector DNA was mixed with insert DNA in excess (insert to vector ratio with regard to molar concentrations ≈ 5:1).

2.5.5 Transformation of bacteria

2.5.5.1 Transformation of *Escherichia coli* by the $CaCl_2$ method

For preparation of competent *E. coli* cells, bacteria were grown aerobically in 200 ml LB medium until reaching an OD_{578} of 0.6 - 0.7. After cooling the cultures on ice for 10 min, 100 ml culture were harvested by centrifugation (4,000 x g; 10 min; 4 °C).

Cells were suspended in 10 ml of ice-cold $CaCl_2$ solution (100 mM $CaCl_2$/ 10 % w/v glycerol). After centrifugation (4,000 x g; 10 min; 4 °C), cells were suspended in 1 ml of ice-cold $CaCl_2$ solution and either used directly for transformation or shock frozen in liquid nitrogen and stored at - 80 °C. For transformation, 1 µg plasmid DNA was mixed with 50 µl of competent *E. coli* cells and placed on ice for 20 min. After heating the cells for 45 sec at 42 °C, cells were cooled down on ice for 2 min. For regeneration of transformed cells, 1 ml LB medium was added and cells were shaken for 1 h at 37 °C. 400 µl of transformed cells were streaked out on a LB medium agar plate with appropriate antibiotics and incubated for 24 - 48 h at 37 °C.

2.5.5.2 Transformation of *Pseudomonas aeruginosa* by diparental mating

E. coli S17 λ-*pir* carries the *tra* gene essential for mobilization of plasmids during conjugation and was used for transfer of plasmid DNA into *P. aeruginosa.* For a mating experiment 1 ml of an *E. coli* S17 λ-*pir* overnight culture carrying the plasmid of interest was mixed with 200 µl of a *P. aeruginosa* overnight culture. After centrifugation (12,000 x g; 1 min; RT) cells were suspended in 50 µl LB and dropped onto a LB agar plate. After drying for 1 h under the clean bench, cells were incubated for 6 h at 37 °C and than suspended in 1 ml LB medium. 200 µl were plated on AB medium agar plates with appropriate antibiotics and incubated at 37 °C for 24 - 48 h.

2.5.6 Sequencing of plasmid DNA

DNA sequences were obtained with an Abi Prism™ 310 Genetic Analyzer in our institute. Required preparatory PCRs with fluorescence-labeled ddNTPs and purification of PCR products were carried out as described by the manufacturer.

2.5.7 Determination of DNA/RNA concentrations

Concentrations and purities of prepared DNA/RNA were determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm. An A_{260} of 1 corresponded to DNA concentrations of 50 µg ml^{-1} and RNA concentrations of 40 µg ml^{-1}.

2.5.8 Separation of DNA/RNA by gel electrophoresis

2.5.8.1 Agarose gel electrophoresis

For analytical separation of DNA fragments, agarose gels consisting of 1 - 1.5 % (w/v) agarose in TAE buffer were prepared. DNA samples were mixed with 6 x DNA loading dye, loaded onto the gel and electrophoretically separated in TAE buffer applying a voltage of 100 v. GeneRuler™ DNA Ladder Mix and MassRuler™ DNA Ladder Mix were used as molecular weight standards according to the manufacturer's instructions. After electrophoresis, gels were incubated in 1 % (w/v) ethidium bromide solution for 10 - 20 min. Finally, DNA was detected via its fluorescence under UV light (λ = 312 nm).

TAE buffer (pH 8.0)	Tris-acetate	40 mM
	EDTA	1 mM
	dissolved in H_2O_{deion}	
6 x DNA loading dye	bromophenol blue	350 µM
	xylene cyanol FF	450 µM
	glycerol	50 % (w/v)
	dissolved in H_2O_{deion}	

For quality check of RNA and northern blot analysis, 1% (w/v) agarose gels in MOPS buffer containing 5 % (v/v) formaldehyde were used (Liberati *et al.*, 2006). Ten µl of prepared RNA (7 - 10 µg) were mixed with 10 µl RNA loading buffer and incubated for 10 min at 65 °C. Samples were loaded onto the gel and electrophoretically separated in MOPS buffer for 4 - 5 h at 4 °C and 100 v. Gels were incubated in an ethidium bromide solution (5 µg ml^{-1} in MOPS) for 5 min and destained overnight in H_2O_{deion}. RNA was detected under UV light (λ = 312 nm).

10 x MOPS buffer (pH 7.4)	MOPS	200 mM
	Na-acetate	50 mM
	EDTA	10 mM
	dissolved in H_2O_{deion}	
RNA loading dye	formamide	6.5 ml
	formaldehyde	1.2 ml
	10 x MOPS	2.0 ml
	sucrose 50 % (w/v)	0.4 ml
	bromophenol blue	350 µM

2.5.8.2 Polyacrylamide gel electrophoresis

For analytical separation of cDNA, 15 % (v/v) polyacrylamide gels in TBE buffer were prepared. cDNA samples were mixed with 6 x DNA loading dye (see 2.5.8.1) and Hyper Ladder™ DNA Ladder was used as size standards according to the manufacturer's instructions. A voltage of 100 was applied. After electrophoresis in TBE buffer for 90 min, gels were incubated in a SYBR gold nucleic stain solution (0.1 % v/v) for 10 min at RT. cDNA was detected under UV light (λ = 312 nm).

5 x TBE buffer (pH 8.3)	Tris	54.0 g
	boric acid	27.5 g
	EDTA (0.5 M; pH 8.0)	20 ml
	H_2O_{deion}	ad 1 l

2.5.9 Northern blot analysis

For northern blotting, electrophoretically separated RNA was transferred to a positively charged nylon membrane by vacuum transfer. RNA gels were first treated with denaturation and neutralization solution for 5 min at RT, respectively. Transfer of RNA onto the membrane was performed with the help of 20 x SSC by vacuum blotting for 4 h at 50 mbar. After blotting, RNA was covalently linked to the membrane by UV light. For hybridization, the membrane was first incubated with 20 ml prehybridization solution for 1 h at 68 °C followed by hybridization with digoxygenin-labeled *arcA*/*nirS* specific probes constructed by Dr. Katharina Trunk from our laboratory overnight at 68 °C (Trunk, 2005). Unspecific bound RNA was removed by washing the membrane three times with washing buffer for 20 min at 68 °C. Prior detection, the membrane was washed with maleic acid buffer for 5 min at RT. After incubation with 5 % (w/v) blocking reagent for 1 h at RT, the blot was incubated with 1.5 µl anti-dig-antibody in 5 % (w/v) blocking reagent for 30 min at RT, washed four times with maleic acid buffer for 10 min at RT and incubated with substrate buffer for 5 min at RT. Detection of RNA was carried out by using 15 µl CDP-Star visible via its chemiluminescence in 1.5 ml substrate buffer.

Denaturation solution	NaOH	0.5 N
	NaCl	1.5 M
	dissolved in H_2O_{deion}	

Neutralization solution	Tris	1.0 M
(pH 7.0)	NaCl	1.5 M
	dissolved in H_2O_{deion}	
20 x SSC (pH 7.0)	Na_3-citrate	300 mM
	NaCl	3.0 M
	dissolved in H_2O_{deion}	
Prehydridization solution	Na_2HPO_4	250 mM
	EDTA	1 mM
	SDS (pH 7.2)	20 % (w/v)
	dissolved in H_2O_{deion}	
Washing buffer	Na_2HPO_4	20 mM
	EDTA	0.1 mM
	SDS (pH 7.0)	1 % (w/v)
	dissolved in H_2O_{deion}	
Maleic acid buffer	maleic acid	100 mM
	NaCl	3 M
	Tween (pH 8.0)	3 % (v/v)
	dissolved in H_2O_{deion}	
Substrate buffer (pH 9.5)	Tris	100 mM
	NaCl	100 mM
	dissolved in H_2O_{deion}	

2.5.10 Procedures for microarray analysis

cDNA synthesis, fragmentation and labeling were performed with minor changes according to protocols of the Affymetrix *Pseudomonas* GeneChip®.

2.5.10.1 Generation of cDNA probes

Ten µg of RNA prepared from three independent experiments were supplemented with 750 ng Random primers and filled up to 30 µl with H_2O_{deion}. An initial denaturation step for 10 min at 70 °C and incubation for 10 min at 25 °C allowed primer annealing. Reverse transcription of RNA was performed by adding 30 µl cDNA reaction mix and the following incubation steps: 10 min at 25 °C, 60 min at 37 °C, 60 min at 42 °C and finally 10 min at 70 °C. Removal of RNA was achieved by addition of 20 µl NaOH (1 N) and heating for 30 min at 65 °C. For neutralization 20 µl HCl (1 N) were added. Samples were purified using the QIAquick PCR Purification Kit.

cDNA reaction mix	5 x 1st strand buffer	12.0 µl
	100 mM DTT	6.0 µl
	dNTPs	3.0 µl
	SUPERase*In (20 U μl^{-1})	1.5 µl
	SuperScript II (200 U μl^{-1})	7.5 µl

Resulting cDNA was partially digested by adding 0.7 U/ µg DNase I and incubation for 5 - 15 min at 37 °C to yield the desired cDNA size range of 50 - 200 bases. The exact incubation period was determined by testing small aliquots of remaining cDNA. DNase I was inactivated by heating the sample for 10 min at 98 °C. Fragmentation was confirmed by polyacrylamide gel electrophoresis. For biotin-ddUTP labeling of cDNA a terminal transferase was used. 33 - 36 µl fragmentized cDNA were added to the labeling reaction mix and incubated for 60 min at 37 °C. Reaction was stopped by the addition of 2 µl EDTA (500 mM).

Labeling reaction mix	5 x reaction buffer	10 µl
	GeneChip® labeling reagent	2 µl
	Terminal transferase (30 U μl^{-1})	2 µl

For verification of labeling efficiency, 5 µl of labeled cDNA were incubated with 5 µl of the biotin binding reagent NeutrAvidine™ (0.4 % w/v in 50 mM Tris) for 5 min at RT. cDNA was then separated by polyacrylamide gel electrophoresis and detected under UV light after SYBR gold staining .

Target hybridization, washing, staining and scanning were performed by the Affymetrix Core Facility at the Helmholtz Centre for Infection Research (HZI), Braunschweig.

2.5.10.2 Microarray data analysis

Raw microarray data were preprocessed with the Bioconductor software framework (Gentleman *et al.*, 2004). Expression values were calculated by the Robust Multichip Average (RMA) method using quantile normalization, background corrected PM intensities and median polish as summarization method (Bolstad *et al.*, 2003; Irizarry *et al.*, 2003a; Irizarry *et al.*, 2003b).

2.6 BIOCHEMICAL TECHNIQUES

2.6.1 Construction and testing of promoter *lacZ* reporter gene fusions

For investigations of promoter activities, the β-galactosidase enzyme assay was used. Active β-galactosidase is able to convert the colorless substrate o-nitrophenyl-galactosid (ONPG) into yellow o-nitrophenolate, which is photometrically detectable by measuring the absorption at 420 nm (A_{420}).

2.6.1.1 Construction of promoter *lacZ* reporter gene fusions

For reporter gene testing, the complete promoter region of interest was fused to *E. coli lacZ*. The promoter was amplified by PCR and the resulting product was digested and cloned into the corresponding restriction site of the digested mini-CTX-*lacZ* vector. Transfer of plasmids into *P. aeruginosa* was carried out by diparental mating as described above resulting in integration of the promoter *lacZ* fusion into the *attB* site of the *P. aeruginosa* genome. In some strains parts of the mini-CTX-*lacZ* containing the tetracycline resistance gene were deleted using a FLP recombinase encoded on the pFLP2 plasmid (Hoang *et al.*, 1998).

2.6.1.2 β-Galactosidase enzyme assay

For determination of promoter activities, the *lacZ* reporter gene fusion carrying strains were incubated as indicated. At defined time points 1 ml of the culture was taken for the β-galactosidase assay. After centrifugation (16,000 x g; 3 min; 4 °C) the cell sediment was frozen at - 20 °C. For enzyme assay, the bacterial sediment was suspended in 600 µl Z-buffer. 250 µl of cell suspension were added to 750 µl H_2O for determining the OD_{578} and 250 µl were mixed with 700 µl Z-buffer, 25 µl chloroform, 25 µl 0.1 % (w/v) SDS, incubated for 5 min at RT and used for enzyme assay. The reaction started by adding 200 µl ONPG (4 mg ml^{-1}) to the suspension and was stopped when the color of the reaction mixture turned yellow by adding 500 µl 1 M sodium carbonate. Enzyme activity was determined by measuring absorption of A_{420}. β-Galactosidase activities are given in Miller Units as an indirect mass of gene expression of the tested promoters. Activities were determined with the following equation:

Miller Units (MU) = A_{420} * 1000/ OD_{578} * v * t

Data are the result of at least three independent experiments.

Z-buffer	Na_2HPO_4	60 mM
	NaH_2PO_2	40 mM
	KCl	10 mM
	$MgSO_4$	1 mM
	β-mercaptoethanol	50 mM
	dissolved in H_2O_{deion}	

2.6.2 Procedures for metabolome analysis

2.6.2.1 Extraction of metabolites

For metabolome analysis, a minimum of four independent experiments was used. *P. aeruginosa* cultures were grown until the desired OD_{578} and harvested at the appropriate time point as indicated. 150 mg cell wet weight (CWW) was harvested by centrifugation (4,000 x g, 20 min and 4 °C). Cells were washed two times with 2 ml 0.9 % (w/v) NaCl and centrifuged (16,000 x g; 2 min; 4 °C). Cells were suspended in 1.5 ml methanol and 7.5 µl ribitol (200 µg ml^{-1}) were added serving as an internal standard. Cells were disrupted by three freeze/thaw cycles of cell suspension at - 80 °C. After centrifugation (16,000 x g, 2 min, 4 °C) supernatant was mixed with 1.5 ml water and 1.5 ml chloroform. After centrifugation (4,000 x g, 10 min, 4 °C) 2 ml of the methanol/water phase were dried in a rotary evaporator overnight at RT.

2.6.2.2 Metabolite derivatization

For derivatization of metabolites, dried samples were treated with 50 µl methoxyamine hydrochloride in pyridine (20 mg ml^{-1}) for 90 min at 30 °C. Samples were then trimethylsilylated for 30 min at 37 °C and 2 h at RT using 80 µl MSTFA (N-methyl-N-(trimethylsilyl)-trifluoroacetamide). Subsequently, 10 µl of time standard containing of eight hydrocarbons (C10, 12, 15, 19, 22, 28, 32 and 36, 2 mg ml^{-1} each) were added for scaling retention indices.

2.6.2.3 Identification and quantification of metabolites

GC-MS analysis has been carried out as described before with a Finnigan Trace gas chromatograph carrying an AS 2000 autosampler and a Finnigan Trace 2000 mass spectrometer (Strelkov *et al.*, 2004; Thielen, 2008). Metabolites were identified by processing the raw GC-MS data with the AMDIS 2.1 software of NIST (National Institute of Standards and Technology, Gaitersburg, USA). For quantitative processing and manual analysis the Quan Browser of the Finnigan Xcalibur software 1.2 (Thermo Electron, Dreieich, Germany) was used.

2.6.2.4 Metabolome data analysis

Quantitative processed metabolome data were analyzed by a statistical likelihood ratio test combined with a universal noise model providing significant differences between two conditions (Klawonn, 2007).

3 RESULTS AND DISCUSSION

3.1 INVESTIGATIONS OF THE KEY REGULATORS REQUIRED FOR ANAEROBIC GROWTH OF *PSEUDOMONAS AERUGINOSA*

In *P. aeruginosa* anaerobic conditions were shown to be important for biofilm growth and infection of the cystic fibrosis (CF) lung (Borriello *et al.*, 2004; Hassett *et al.*, 2002; Palmer *et al.*, 2007; Worlitzsch *et al.*, 2002; Yoon *et al.*, 2002). Anaerobic growth of *P. aeruginosa* is possible via arginine fermentation or denitrification (Vander Wauven *et al.*, 1984; Zumft, 1997). In the presence of nitrate or nitrite, *P. aeruginosa* performs a highly efficient denitrification. Expression of genes encoding the enzymes required for denitrification depends on the transcriptional regulators Anr (anaerobic regulation of arginine deiminase and nitrate reduction) and Dnr (dissimilatory nitrate respiration regulator) as well as the nitrate sensing two-component system NarX-NarL (Arai *et al.*, 1997; Schreiber *et al.*, 2007; Ye *et al.*, 1995). Transcription of the *arcDABC* operon encoding the enzymes essential for arginine fermentation depends on Anr and is further stimulated by the arginine responsive regulator ArgR (Lu *et al.*, 1999; Vander Wauven *et al.*, 1984).

Recent publications illustrated the global transcriptional response of *P. aeruginosa* to anaerobic denitrifying growth conditions by the use of microarrays (Filiatrault *et al.*, 2005; Platt *et al.*, 2008). Our laboratory investigated the key regulators of anaerobic growth in detail and identified genes whose expression is controlled by Anr, Dnr and NarX-NarL showing that Anr is the major regulator of anaerobic denitrifying growth (Quäck, 2005; Trunk, 2005). Based on these results from our laboratory, in the first part of this work the key regulators of the anaerobic regulatory network were further investigated with the help of microarrays, northern blot analysis and *lacZ* reporter gene studies.

3.1.1 Identification of genes regulated by the nitrate response regulator NarL in *Pseudomonas aeruginosa* using microarrays

Recent investigations of the *E. coli* NarX-NarL two-component system by a transcriptomic analysis revealed the NarL-dependent expression of more than 90 operons (Constantinidou *et al.*, 2006). In contrast, only a few genes were shown to be targets of the two-component response regulator NarL in *P. aeruginosa* including

dnr, *hemA*, *narK$_1$K$_2$GHJI* and *nirQ* (Krieger *et al.*, 2002; Schreiber *et al.*, 2007). Direct activation of genes by NarL via binding to a conserved NarL binding site in the promoter region of target genes was shown for the nitrate reductase operon *narK$_1$K$_2$GHJI* and *hemA* using promoter *lacZ* reporter gene fusions and by site directed mutagenesis of the respective binding sites (Krieger *et al.*, 2002; Schreiber *et al.*, 2007).

In order to identify new members of the *P. aeruginosa* NarL regulon, a comprehensive transcriptomic approach was applied. Expression profiles of the *P. aeruginosa* wild type PAO1 and the *narL* knockout mutant PAO9104 were analyzed using the Affymetrix *Pseudomonas* GeneChip® (Krieger *et al.*, 2002).

3.1.1.1 Experimental set up for microarray analyses

Since the *narL* mutant PAO9104 cannot grow in the absence of oxygen, shift experiments were performed. Therefore, *P. aeruginosa* cells were grown in 100 ml LB medium in sealed serum flasks allowing aerobic growth for three hours up to an OD_{578} of 0.2. Then growth stopped and cells adapted to anaerobic conditions. Only when nitrate was present as electron acceptor, *P. aeruginosa* wild type cells resumed growth after 4 hours, see fig. 3.1.

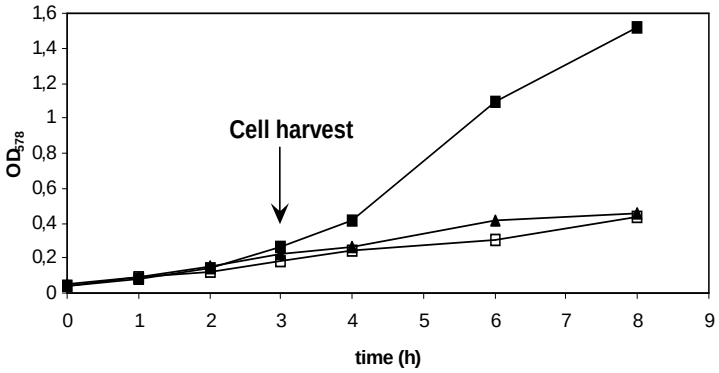


Figure 3.1: Growth curve of *P. aeruginosa* under oxygen limitation.

Cells were grown in LB medium under oxygen limited conditions at 37 °C. Filled squares: PAO1 in LB plus 50 mM KNO_3; open squares: PAO1 in LB without KNO_3; filled triangles: *narL* mutant in LB plus KNO_3.

To confirm anaerobic conditions, northern blot analysis was applied using a *nirS* specific probe as expression of *nirS* is directly activated by Dnr and thus requires an

active Anr-Dnr regulatory cascade. Therefore, *P. aeruginosa* wild type cells were incubated in LB medium supplemented with 50 mM nitrate and in LB medium without the addition of nitrate for 3 h at 37 °C. R NA was extracted and subjected for northern blot analysis as described in Materials and Methods.

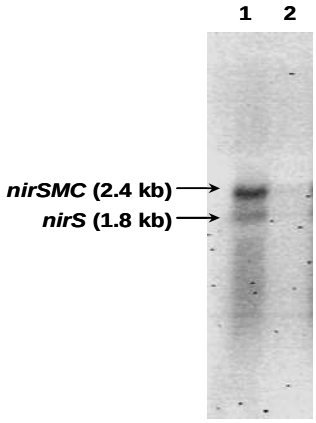


Figure 3.2: Northern blot analysis of *nirSMC* mRNA. Northern blot analysis with RNA prepared from *P. aeruginosa* wild type PAO1 grown under oxygen limitation in LB medium with 50 mM (lane 1) and the wild type PAO1 grown without KNO_3 (lane 2). Total RNA (10 µg) was isolated after 3 h of incubation at 37 °C and hybridized with a *nirS* specific probe (Trunk, 2005).

A 2.4 kb *nirSMC* transcript and a 1.8 kb *nirS* transcript were only detectable for the wild type incubated with nitrate as described elsewhere, see fig. 3.2 (Trunk, 2005). This clearly confirmed anaerobic denitrifying conditions after 3 h of incubation, since expression of the *nir* operon is only activated in response to anaerobic denitrifying conditions by Dnr (Arai *et al.*, 1995a).

Consequently, for microarray analyses cells were grown in LB medium under the tested condition and RNA was extracted after three hours, when all cells showed the same growth behavior during adaptation to anaerobic growth conditions. RNA extraction, cDNA synthesis, fragmentation and cDNA labeling were performed as described in Materials and Methods. Target hybridization, washing, staining and scanning of the GeneChips® were performed by the Affymetrix Core Facility at the Helmholtz Centre for Infection Research (HZI), Braunschweig. Microarray experiments were carried out in triplicate for the *P. aeruginosa* wild type strain PAO1 grown anaerobically in LB medium in the presence of 50 mM nitrate, 10 mM nitrite and in the absence of any electron acceptors. The *narL* mutant PAO9104 was grown in LB with 50 mM nitrate. For analysis of the transcriptome data the Bioconductor software package was used as described in Material and Methods and elsewhere (Bolstad *et al.*, 2003; Gentleman *et al.*, 2004; Irizarry *et al.*, 2003a; Irizarry *et al.*, 2003b).

3.1.1.2 Initial transcriptional response of *Pseudomonas aeruginosa* to anaerobic denitrifying conditions

For identification of genes involved in denitrification, first transcription profiles of the wild type PAO1 grown under oxygen limited conditions in the presence of nitrate were compared with the expression profile of *P. aeruginosa* cells grown without the addition of nitrate. A total of 182 genes were found differentially expressed in response to nitrate representing 3.3 % of the genome. Among these genes, 115 were upregulated and 67 were downregulated more than twofold during anaerobic growth with nitrate.

Functional classification according to the Pseudomonas database showed that most of the genes induced during anaerobic denitrifying growth are involved in energy metabolism, see fig. 3.3 (Winsor *et al.*, 2005; Winsor *et al.*, 2009). Genes encoding proteins catalyzing the biosynthesis of cofactors were also found highly induced by nitrate. No repression of genes belonging to this functional class could be observed. In addition, genes involved in antibiotic resistance, central intermediary metabolism, chaperones, heat-shock and nucleotide biosynthesis were found to be activated by nitrate. The majority of genes found downregulated and many genes found upregulated in response to nitrate encode hypothetical proteins with unknown function. Further, genes encoding proteins for motility and attachment and genes related to phage, transposon or plasmid were found repressed by nitrate.

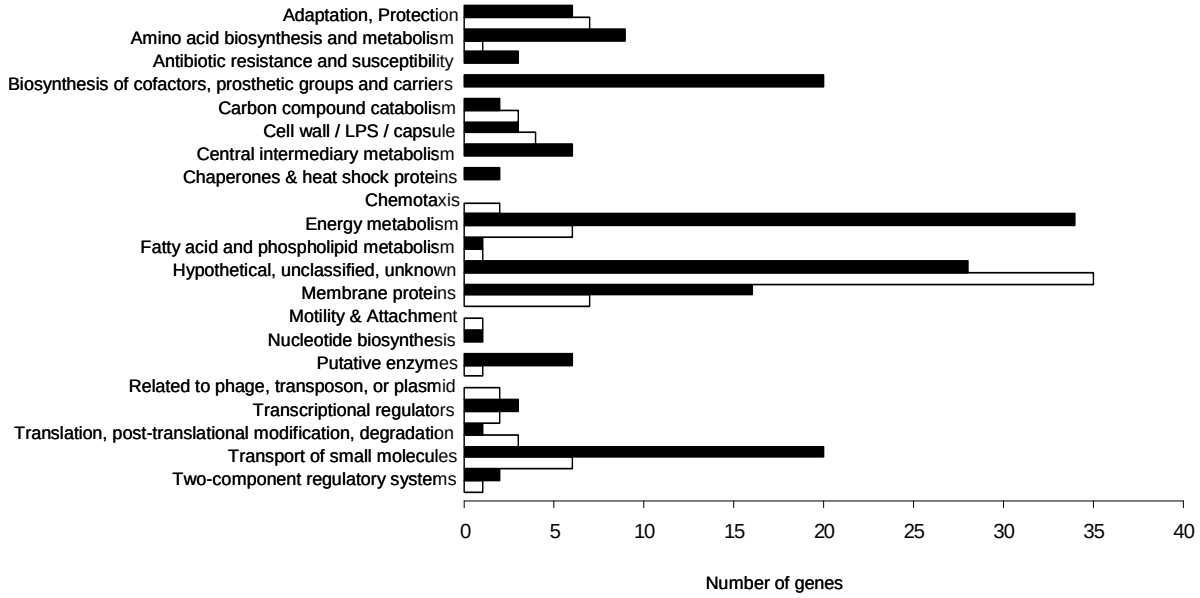


Figure 3.3: Functional classification of genes differentially expressed in response to nitrate.
Genes are classified according to the Pseudomonas database (www.pseudomonas.com). Black: induced by nitrate; white: repressed by nitrate.

A list of genes differentially expressed between the *P. aeruginosa* wild type grown with nitrate and without the addition of nitrate (Wt + NO_3^- versus Wt - NO_3^-) is given in tab. 3.1. Further comparison of transcription profiles of wild type cells grown with nitrate to cells incubated with nitrite (Wt + NO_3^- versus Wt + NO_2^-) allowed discrimination of gene expression depending on nitrate, nitrite or both, see tab. 3.1.

Table 3.1: Differential gene expression of *P. aeruginosa* under oxygen limited conditions in response to nitrate and to nitrite.

Gene ID[a]	Gene name[a]	Operon structure[a]	Function[a]	Ratio[b] + NO_3^- / - NO_3^-	+ NO_3^- / + NO_2^-
PA0524	*norB*	*norCBD*	nitric-oxide reductase subunit B	130.29	
PA3874	*narH*	*narK$_1$K$_2$GHJI*	respiratory nitrate reductase beta chain	54.47	9.26
PA3392	*nosZ*	*nosRZDFYL*	nitrous-oxide reductase precursor	36.55	
PA1856		*PA1855-1856*	probable cytochrome oxidase subunit	27.89	
PA3871	*nifM*		probable peptidyl-prolyl cis-trans isomerase, PpiC-type	24.09	8.4
PA3915	*moaB$_1$*	*moaB$_1$-moeA$_1$*	molybdopterin biosynthetic protein B1	20.00	9.52
PA1854			conserved hypothetical protein	17.43	2.02
PA3870	*moaA$_1$*		molybdopterin biosynthetic protein A1	15.33	9.29
PA2664	*fhp*		flavohemoprotein	14.92	
PA2663		*PA2662-2663*	hypothetical protein	12.06	
PA0513	*nirG*	*nirSMCFDLGHJEN*	probable transcriptional regulator	11.10	
PA0521	*nirO*	*nirQOP*	probable cytochrome c oxidase subunit	10.50	
PA3912	*yhbV*	*PA3911-3913*	conserved hypothetical protein	7.62	2.06
PA0526			hypothetical protein	4.93	

Table 3.1 continued

Gene ID[a]	Gene name[a]	Operon structure[a]	Function[a]	Ratio[b] + NO_3^- / - NO_3^-	+ NO_3^- / + NO_2^-
PA3530			conserved hypothetical protein	4.00	
PA4773		*PA4773-4775-pmrAB*	hypothetical protein	3.94	10.61
PA4205	*mexG*	*mexGHI-opmD*	hypothetical protein	3.93	
PA4812	*fdnG*	*fdnGHI*	formate dehydrogenase-O, major subunit	3.92	2.68
PA3880			conserved hypothetical protein	3.81	
PA0291	*oprE*		anaerobically-induced outer membrane porin OprE precursor	3.78	4.82
PA4771	*lldD*		L-lactate dehydrogenase	3.71	4.14
PA1337	*ansB*		glutaminase-asparaginase	3.69	2.15
PA4515	*piuC*	*piuCAB*	conserved hypothetical protein	3.63	3.52
PA1421	*gbuA*		guanidinobutyrase	3.56	4.06
PA2204			probable binding protein component of ABC transporter	3.46	10.09
PA5531	*tonB*		TonB protein	3.38	
PA3553	*arnC*	*arnBCADTEF-PA3559*	ArnC	3.36	4.46
PA0283	*sbp*		sulfate-binding protein precursor	3.28	3.73
PA1838	*cysI*	*cysI-PA1837*	Sulfite reductase	3.25	8.11
PA0782	*putA*	*putAP*	proline dehydrogenase PutA	3.25	2.29
PA4809	*fdhE*		FdhE protein	3.20	
PA4770	*lldP*		L-lactate permease	3.19	3.12
PA2398	*fpvA*		ferripyoverdine receptor	3.05	2.56
PA3918	*moaC*	*moaCDE*	molybdopterin biosynthetic protein C	3.04	3.35
PA0024	*hemF*	*hemF-aroE*	coproporphyrinogen III oxidase, aerobic	2.98	
PA4443	*cysD*	*cysDN*	ATP sulfurylase small subunit	2.97	7.83
PA2691			conserved hypothetical protein	2.92	0.49
PA5373	*betB*	*betIBA*	betaine aldehyde dehydrogenase	2.89	
PA2761			hypothetical protein	2.83	
PA5024			conserved hypothetical protein	2.82	3.01
PA0284			hypothetical protein	2.81	8.37
PA4687	*hitA*	*hitAB*	ferric iron-binding periplasmic protein HitA	2.73	3.27
PA2274			hypothetical protein	2.72	
PA1340		*PA1339-1342*	probable permease of ABC transporter	2.71	2.41
PA3814	*iscS*	*iscSUA*	L-cysteine desulfurase (pyridoxal phosphate-dependent)	2.62	
PA4371			hypothetical protein	2.59	2.18
PA5496	*nrdJb*	*ndrJab*	class II (cobalamin-dependent) ribonucleotide-diphosphate reductase subunit, NrdJb	2.54	
PA3811	*hscB*	*hscBA*	heat shock protein HscB	2.45	
PA0130			probable aldehyde dehydrogenase	2.43	
PA1420			hypothetical protein	2.36	2.16
PA3205			hypothetical protein	2.35	
PA1559		*PA1559-1560*	hypothetical protein	2.30	6.36
PA0795	*prpC*	*PA0797-prpBC-PA0794*	citrate synthase 2	2.25	
PA5217			probable binding protein component of ABC iron transporter	2.20	
PA4782		*PA4783-4782*	hypothetical protein	2.12	3.91
PA2532	*tpx*		thiol peroxidase	2.02	
PA3268			probable TonB-dependent receptor	2.02	
PA0665		*argC-PA0663-0664*	conserved hypothetical protein	2.01	

Table 3.1 continued

Gene ID[a]	Gene name[a]	Operon structure[a]	Function[a]	Ratio[b] + NO_3^- / - NO_3^-	+ NO_3^-/ + NO_2^-
PA3142		*PA3144-3142*	hypothetical protein	0.50	
PA3531	*bfrB*		bacterioferritin	0.50	
PA3152	*hisH*$_2$	*hisF*$_2$-*hisH*$_2$	glutamine amidotransferase	0.50	
PA3183	*zwf*		glucose-6-phosphate 1-dehydrogenase	0.50	
PA3144			hypothetical protein	0.49	
PA1747			hypothetical protein	0.49	
PA2128	*cupA1*		fimbrial subunit CupA1	0.49	
PA5231	*yhiH*		probable ATP-binding/permease fusion ABC transporter	0.49	
PA3155	*wbpE*	*wbpCDE*	probable aminotransferase WbpE	0.49	
PA1561	*aer*		aerotaxis receptor Aer	0.48	
PA3904		*PA3904-3908*	hypothetical protein	0.48	
PA2127			conserved hypothetical protein	0.48	
PA0459	*clpC*		probable ClpA/B protease ATP binding subunit	0.48	
PA1324			hypothetical protein	0.48	
PA1544	*anr*		transcriptional regulator Anr	0.47	
PA5232	*yhiI*	*PA5232-5230*	conserved hypothetical protein	0.47	
PA3366	*amiE*		aliphatic amidase	0.47	
PA5427	*adhA*		alcohol dehydrogenase	0.46	
PA5027	*uspO*		hypothetical protein	0.46	
PA3930	*cioA*	*cioAB*	cyanide insensitive terminal oxidase	0.46	
PA4348			conserved hypothetical protein	0.46	
PA1869			probable acyl carrier protein	0.46	0.25
PA3150	*wbpG*	*wbpGHI*	LPS biosynthesis protein WbpG	0.46	
PA1123			hypothetical protein	0.46	
PA2754			conserved hypothetical protein	0.46	
PA1323			hypothetical protein	0.46	
PA4328	*uspM*		universal stress protein	0.46	
PA3040	*yqjD*		conserved hypothetical protein	0.45	
PA1557		*PA1555-1557*	probable cytochrome oxidase subunit (cbb3-type)	0.45	
PA3692			probable outer membrane protein precursor	0.45	
PA4917		*PA4917-4916*	hypothetical protein	0.45	
PA4587	*ccpR*		cytochrome c551 peroxidase precursor	0.45	
PA1635	*kdpC*	*kdpABCDE*	potassium-transporting ATPase, C chain	0.45	0.44
PA3160	*wzz*		O-antigen chain length regulator	0.44	
PA3691			hypothetical protein	0.44	
PA1852			hypothetical protein	0.44	0.40
PA4614	*mscL*		conductance mechanosensitive channel	0.43	
PA0731			hypothetical protein	0.43	
PA3326			probable Clp-family ATP-dependent protease	0.43	0.30
PA0039			hypothetical protein	0.43	0.43
PA0179		*PA0179-0177*	probable two-component response regulator	0.43	0.45
PA3476	*rhlI*		autoinducer synthesis protein RhlI	0.43	0.46
PA1151	*imm2*		pyocin S2 immunity protein	0.43	
PA2501			hypothetical protein	0.42	
PA3575			hypothetical protein	0.42	
PA3479	*rhlA*	*rhlAB*	rhamnosyltransferase chain A	0.42	0.47
PA1789	*uspL*		hypothetical protein	0.42	
PA0588	*yeaG*		conserved hypothetical protein	0.41	
PA5460			hypothetical protein	0.39	
PA3496			hypothetical protein	0.38	0.46
PA2482		*PA2482-2481*	probable cytochrome c	0.38	

Table 3.1 continued

Gene ID[a]	Gene name[a]	Operon structure[a]	Function[a]	Ratio[b] + NO_3^- / - NO_3^-	+ NO_3^-/ + NO_2^-
PA3337	*rfaD*		ADP-L-glycero-D-mannoheptose 6-epimerase	0.38	
PA1051			probable transporter	0.38	0.42
PA0805			hypothetical protein	0.37	
PA4607			hypothetical protein	0.37	0.36
PA5475			hypothetical protein	0.36	
PA4377			hypothetical protein	0.35	
PA3431	*ywbG*	*PA3432-3431*	conserved hypothetical protein	0.34	0.46
PA4141			hypothetical protein	0.32	0.21
PA4611			hypothetical protein	0.32	
PA0141			conserved hypothetical protein	0.30	
PA2622	*cspD*		cold-shock protein CspD	0.29	
PA0128	*phnA*		conserved hypothetical protein	0.28	
PA3049	*rmf*		ribosome modulation factor	0.26	0.20
PA3284		*PA3284-3281*	hypothetical protein	0.18	
PA1746			hypothetical protein	0.11	0.47

[a] Gene ID, gene name, function and operon structure are according to the Pseudomonas database (www.pseudomonas.com). Only the gene with the highest average expression ratio of a putative operon is shown.

[b] Ratio means fold change between two conditions of at least < 0.5 and > 2. The wild type PAO1 grown under oxygen limited conditions in LB medium containing 50 mM KNO_3 was compared with the wild type grown without the addition of nitrate and with the wild type incubated with 10 mM $NaNO_2$. Where no ratio is shown, no changes in gene expression were detected.

Consistent with recently published microarray data of *P. aeruginosa*, the highest induced genes during anaerobic denitrification belong to the *norCBD*, the *narK$_1$K$_2$GHJI*, the *nirSMCFDLGHJEN* and the *nosRZDFYL* operon encoding the four reductases and the *nirQOP* operon all essential for denitrification (Alvarez-Ortega and Harwood, 2007; Filiatrault *et al.*, 2005; Platt *et al.*, 2008). As expected, the *nir*, *nor* and *nos* genes encoding enzymes essential for nitrite respiration were induced in response to nitrate and nitrite. However, the *nar* operon required nitrate for full induction implying that the two-component system NarX-NarL is activated preferentially by nitrate than nitrite, which is similar to the homologous *E. coli* NarX-NarL system (Williams and Stewart, 1997). Expression of *narXL* itself in response to the tested conditions remained unchanged. This was previously shown in our group by *lacZ* reporter gene experiments (Schreiber *et al.*, 2007).

Furthermore, genes encoding proteins catalyzing the molybdopterin guanine dinucleotide cofactor biosynthesis *moaA1, moaB1, moeA1* and *moaCDE* were found highly activated in response to nitrate, which was also previously described for *P. aeruginosa* and additionally for *E. coli* (Constantinidou *et al.*, 2006; Filiatrault *et al.*,

2005; Platt *et al.*, 2008). In agreement, molybdenum cofactor biosynthesis is required for nitrate reductase activity in *P. aeruginosa* (Noriega *et al.*, 2005). Moreover, expression of the *nifM* gene encoding peptidyl-prolyl *cis/trans* isomerase described to function in maturation of an iron-sulfur cluster containing protein was found activated during nitrate respiration (Gavini *et al.*, 2006). Consistent with previously published data, full expression of these genes was observed in the presence of nitrate indicating a possible NarL-dependence (Platt *et al.*, 2008).

Expression of *fhp* encoding a flavohemoprotein was found induced in the presence of nitrate as well as nitrite which was also previously described (Arai *et al.*, 2005). Under aerobic conditions Fhp is suggested to normally function in detoxification of NO and under anaerobic conditions it was proposed that expression of *fhp* may be beneficial for survival at the aerobic/anaerobic interface (Arai *et al.*, 2005). Unexpectedly, increased expression of the PA4773-4775-*pmrAB* operon was detected under anaerobic denitrifying conditions. This operon consists of the PmrA-PmrB two-component system, which was found induced in response to low magnesium conditions and in the presence of cationic antimicrobial peptides (McPhee *et al.*, 2006; Moskowitz *et al.*, 2004). Moreover, transcription of the *mexGHI-opmD* operon facilitating quorum sensing, antibiotic resistance and promoting virulence and growth in *P. aeruginosa* was found induced during denitrification (Aendekerk *et al.*, 2005). Transcription of these genes is controlled by PmrA-PmrB and by the *P. aeruginosa* transcriptional regulator SoxR activating the oxidative stress adaptive response (McPhee *et al.*, 2006; Palma *et al.*, 2005). In contrast to the PmrA-PmrB regulated genes, the two-component system itself is more activated during nitrate respiration than nitrite respiration.

In agreement with published data about the *E. coli fdnGHI* operon encoding a formate dehydrogenase, transcription of the *P. aeruginosa fdnGHI* genes as well as the *fdhE* gene encoding the FdhE protein was found induced during denitrification (Wang and Gunsalus, 2003). It was proposed, that the *P. aeruginosa* formate dehydrogenase directly transfers electrons taken from formate oxidation to the *nar* nitrate reductase without passing quinones, which is in contrast to *E. coli*, where electrons are transferred to the nitrate reductase via quinones (Williams *et al.*, 2007). Interestingly, transcription of the *tonB* gene was found increased under denitrifying conditions. TonB was previously shown to couple the proton motive force (PMF) of the inner membrane to the active siderophore uptake across the outer membrane

and is induced under iron limiting conditions (Bayer and Harjes, 2001). Moreover, expression of *fpvA* encoding a ferripyoverdine receptor involved in iron uptake into the cell and *hitAB* encoding a ferric iron-binding periplasmic protein were also upregulated in response to denitrifying conditions (Poole *et al.*, 1993). Transcription of these genes ensures uptake of siderophores and subsequently the availability of iron in the cell. In this context, expression of *cysDN, cysI* and *sbp,* all encoding proteins involved in uptake and reduction of sulfate was found induced in the *P. aeruginosa* wild type incubated with nitrate. Induction of all these genes suggests a higher demand of iron and sulfur under anaerobic denitrifying conditions, which might be caused by the biosynthesis of iron-sulfur cluster containing proteins. In agreement, transcription of the *iscSUA* and *hscBA* operon was found upregulated in response to denitrifying conditions. It was proposed, that these genes contribute to the formation of the active site of iron-sulfur cluster containing proteins (Campos-Garcia *et al.*, 2000). In *E. coli*, it was shown that *iscSUA* and *hscBA* are important for Fe-S cluster assembly of Fnr upon the onset of anaerobic growth conditions (Mettert *et al.*, 2008).

Upregulation of the *hemF-aroE* gene cluster encoding the aerobic coproporphyrinogen III oxidase during denitrification is in accordance with published data showing that *hemF* expression is controlled by the dual action of the global anaerobic regulator Anr and the N-oxide regulator Dnr under anaerobic denitrifying conditions (Rompf *et al.*, 1998).

Genes involved in B-band LPS biosynthesis, the *wbpCDE* and *wbpGHI* operons (Rocchetta *et al.*, 1999), were found repressed during denitrification. It has been shown, that the amount of B-band LPS from *P. aeruginosa* decreases under oxygen limitation (Sabra *et al.*, 2003). In addition, two genes important for LPS biosynthesis, *rfaD* and *wzz*, were also repressed by nitrate/nitrite indicating differences in LPS composition during denitrifying growth.

Transcription of the *aer* gene encoding an aerotaxis receptor protein involved in sensing oxygen was found downregulated in the presence of nitrate/nitrite. As a result of energy starvation caused by the absence of electron acceptors, expression of genes encoding electron acceptor detecting systems is activated (Nichols and Harwood, 2000). In *P. aeruginosa*, aerotaxis was found induced upon entry into stationary phase and moreover, expression of the *aer* gene was shown to be regulated by the global oxygen regulator Anr (Hong *et al.*, 2004).

A ClpC-type protease gene encoded by PA0459 and *cspD* encoding a cold shock protein as well as another putative Clp protease encoded by PA3326 were additionally found repressed under denitrifying conditions indicating induction of these genes in response to the absence of electron acceptors and growth arrest. In this context, expression of *rmf* encoding the ribosome modulation factor was found decreased in the presence of nitrate/ nitrite. Since transcription of *rmf* requires the alarmone ppGpp (Izutsu *et al.*, 2001), this indicated stringent response during oxygen limitation without external electron acceptors.

Interestingly, expression of three universal stress proteins, *uspL*, *uspM* and *uspO,* was found decreased in the presence of nitrate indicating an important role in adaptation to energy limited conditions. Transcription of these genes was shown to be regulated by Anr under anaerobic conditions as well as by the stringent response regulators RelA and SpoT during stationary phase (Boes *et al.*, 2008). This is in agreement with repression of *rmf* in the presence of nitrate.

Furthermore, transcription of *adhA* encoding an alcohol dehydrogenase and of genes coding for proteins involved in adaptation and protection was found induced in the absence of nitrate/nitrite under oxygen limited conditions.

Anaerobic repression of quorum sensing (QS) genes, *rhlI* and the *rhlAB,* by nitrate implied an interconnection of denitrification and QS. QS is a bacterial communication system, which was shown to be important for the production of virulence factors and biofilm formation (Van Delden and Iglewski, 1998). Using microarray analysis, expression of genes involved in denitrification, *e. g. nar* and *nos*, was found repressed by QS (Wagner *et al.*, 2003). Moreover, the authors observed decreased expression of *rhlR*, the transcriptional regulator of the Rhl QS system, and *rhlI*, the corresponding autoinducer synthesis, under anaerobic conditions in the presence of nitrate (Wagner *et al.*, 2003). Additionally, repression of denitrification by C4-homoserine lactone, the product of *rhlI*, was recently described (Toyofuku *et al.*, 2007). All these results pointed to complex interaction between the QS and denitrification networks.

Expression of genes coding for a cytochrome *c* oxidase (PA1555-1557) and the Anr-dependent cyanide insensitive terminal oxidase encoding gene *cioA* was also found repressed by nitrate. In agreement, under low oxygen conditions transcription of both oxidases was shown to be induced in the absence of nitrate (Alvarez-Ortega and Harwood, 2007). Further, the cytochrome *c* peroxidase encoding gene *ccpR*

was additionally found repressed in the presence of nitrate. CcpR catalyzes the reduction of hydrogen peroxide to water using cytochrome *c* as electron donor and was also shown to be expressed in response to low oxygen conditions (Alvarez-Ortega and Harwood, 2007; Williams *et al.*, 2007).

3.1.1.3 The *Pseudomonas aeruginosa* NarL regulon

Microarray data on the transcriptional response of *P. aeruginosa* to nitrate and nitrite allowed the identification of nitrate-dependent genes as shown above (tab. 3.1). Expression of these genes might be regulated via nitrate or by the nitrate responsive two-component system NarX-NarL, either directly or indirectly.

To identify NarL-dependent genes, expression profiles of the wild type grown under oxygen limitation supplemented with nitrate and without the addition of nitrate were compared to the *narL* mutant grown in the presence of nitrate. NarL-dependent genes were expected to be expressed only in the wild type incubated with nitrate. No induction of NarL-dependent genes was expected in the *narL* mutant strain as well as in the wild type grown without the addition of nitrate, which prevents activation of the two-component system NarX-NarL. This comparison led to the identification of 42 NarL-dependent genes. Among these genes, 32 were found induced and 10 repressed by NarL as shown in tab. 3.2.

Table 3.2: NarL-dependent gene expression in *P. aeruginosa*.

Gene ID[a]	Gene name[a]	Operon structure[a]	Function[a]	Ratio[b] Wt + NO_3^-/ *narL*$^-$ + NO_3^-
PA3874	*narH*	*narK$_1$K$_2$GHJI*	respiratory nitrate reductase beta chain	47.86
PA3871	*nifM*		probable peptidyl-prolyl cis-trans isomerase, PpiC-type	24.54
PA3915	*moaB$_1$*	*moaB$_1$-moeA$_1$*	molybdopterin biosynthetic protein B1	19.98
PA3870	*moaA$_1$*		molybdopterin biosynthetic protein A1	15.18
PA1854			conserved hypothetical protein	15.04
PA0782	*putA*	*putAP*	proline dehydrogenase PutA	5.48
PA0291	*oprE*		anaerobically induced outer membrane porin OprE precursor	4.29
PA1421	*gbuA*		guanidinobutyrase	3.39
PA2398	*fpvA*		ferripyoverdine receptor	3.36
PA2204			probable binding protein component of ABC transporter	3.30
PA1838	*cysI*	*cysI- PA1837*	sulfite reductase	2.66
PA3918	*moaC*	*moaCDE*	molybdopterin biosynthetic protein C	2.60
PA1337	*ansB*	*ansB*	glutaminase-asparaginase	2.54
PA4777	*pmrB*	*PA4773-4775-pmrAB*	two-component regulator system signal sensor kinase PmrB	2.51
PA1420			hypothetical protein	2.38

Table 3.2 continued

Gene ID[a]	Gene name[a]	Operon structure[a]	Function[a]	Ratio[b] Wt + NO_3^-/ *narL*$^-$ + NO_3^-
PA5024	*ytnM*		conserved hypothetical protein	2.26
PA1559		*PA1559-1560*	hypothetical protein	2.16
PA4771	*lldD*		L-lactate dehydrogenase	2.12
PA4442	*cysN*	*cysDN*	ATP sulfurylase GTP-binding subunit/APS kinase	2.04
PA3479	*rhlA*	*rhlAB*	rhamnosyltransferase chain A	0.50
PA3326			probable Clp-family ATP-dependent protease	0.50
PA0039			hypothetical protein	0.49
PA3496			hypothetical protein	0.48
PA3476	*rhlI*		autoinducer synthesis protein RhlI	0.47
PA1051			probable transporter	0.38
PA0179			probable two-component response regulator	0.37
PA4607			hypothetical protein	0.28
PA4141			hypothetical protein	0.26
PA3049	*rmf*		ribosome modulation factor	0.22

[a] Gene ID, gene name, function and operon structure are according to the Pseudomonas database (www.pseudomonas.com). Only the gene of a putative operon with the highest expression ratio is shown.

[b] Ratio means fold change between the wild type PAO1 and the *narL* mutant PAO9104 both incubated under oxygen limited conditions in LB with 50 mM KNO_3.

As expected from previous work in our laboratory, transcription of the nitrate reductase operon *narK$_1$K$_2$GHJI* clearly depends on NarL (Schreiber *et al.*, 2007). The two following downstream genes *nifM* (PA3871) and *moaA$_1$* were additionally found highly induced in the wild type compared to the *narL* mutant. Further, transcription of the molybdopterin guanine dinucleotide cofactor biosynthesis genes *moaB$_1$*, *moeA$_1$* and *moaCDE* was found to be NarL-dependent. In agreement, molybdopterin guanine dinucleotide is an obligate cofactor for nitrate reductase activity (Philippot and Hojberg, 1999).

Transcription of *ansB*, *gbuA*, *putA* and *putP*, all involved in amino acid catabolism, was also identified as NarL-dependent. NarL activated expression of *ansB* encoding a glutaminase-asparaginase is in contrast to the homologous *E. coli ansB*, which was described as NarL-repressed (Constantinidou *et al.*, 2006). The *gbuA* gene encodes a guanidinobutyrase contributing to arginine degradation and the *putAP* operon encodes a proline dehydrogenase and a proline transporter involved in proline degradation (Nakada and Itoh, 2002; Nakada *et al.*, 2002). The proline dehydrogenase is a flavoprotein associated with the membrane transferring electrons from proline to respiratory chains and thus contributes to proton gradient

formation (Nakada *et al.*, 2002). However, both amino acids, arginine and proline, can be utilized as sole carbon and energy source in *P. aeruginosa* respiration. In this context, *lldD* encoding a lactate dehydrogenase was found induced during denitrification. NarL-dependent activation of these genes implied a potential role under *P. aeruginosa* nitrate respirative conditions.

Consistent with proteome data from our laboratory, expression of *oprE* encoding the porin protein E1 was found NarL-dependent (Benkert *et al.*, 2008). Previously, it was shown that *oprE* is expressed in response to anaerobiosis (Yamano *et al.*, 1993).

Furthermore, activation of *fpvA*, *cysDN* and *cysI-PA1837*, all involved in uptake of iron and sulfate, was found depended on NarL. This leads to the conclusion, that NarL coordinates gene expression of iron and sulfate uptake systems required for iron-sulfur cluster containing enzymes catalyzing respiration. Interestingly, differences in expression levels of the *cys* genes and another putative operon PA1559-1560 encoding hypothetical proteins were found higher between the wild type grown with nitrate compared to the wild type incubated with nitrite than compared to cells grown without nitrate or nitrite, see tab. 3.1. The same observation was made by comparing the *narL* mutant with the wild type grown with nitrite (data not shown) implying a repression of these genes by nitrite independently of NarL.

Repression of *rhlA* encoding the rhamnosyltransferase by NarL is in accordance with the hyper-swarming phenotype of a *narL* mutant strain described in a recent publication (Van Alst *et al.*, 2007). In this study it was proposed, that the absence of NarL leads to an overproduction of the *rhlAB* operon and thus influences biofilm formation. The presence of a putative NarL binding site found in the promoter region of *rhlAB* indicates a direct repression by NarL (Van Alst *et al.*, 2007). Additionally, the *rhlI* gene, which encodes the autoinducer synthesis protein of the Rhl quorum sensing system, is repressed by NarL. Outlined observations in combination with this finding suggest a close and complex interaction between the denitrification and quorum sensing regulatory networks partly mediated by NarL.

In summary, the *P. aeruginosa* NarL regulon consists of more than 40 genes. The majority of NarL-dependent genes are generally involved in energy generation and the biosynthesis of cofactors. NarL positively controls the expression of the nitrate reductase catalyzing the first step in denitrification and the production of cofactors essential for enzyme activity including iron, sulfur and molybdopterin. Moreover,

NarL negatively influences the *P. aeruginosa* quorum sensing system Rhl by repressing the corresponding genes.

Comparison of the *P. aeruginosa* and *E. coli* NarL regulons revealed only little overlap as expected from the different metabolism and regulatory networks (Constantinidou *et al.*, 2006). Only a few orthologous genes are found to be upregulated by NarL in both organisms. These are the membrane-bound nitrate reductase operon *narGHJI* and *moeA/moeA*$_1$. In contrast to the *E. coli ansB* gene found repressed by NarL, the *P. aeruginosa* homologue is activated by NarL. However, in *E. coli* NarL activates transcription of *fdnGHI, lldP* and *cspD* while in *P. aeruginosa* these genes are activated by nitrate independently of NarL.

Note that NarL-dependent induction of *dnr*, *hemA* and *nirQ* and NarL-dependent repression of *arcDABC* was not detected in this analysis, which might be due to low signal intensities.

3.1.2 NarX-NarL mediated repression of arginine fermentation in *P. aeruginosa*

Proteome analysis via 2D gel electrophoresis of the *P. aeruginosa* wild type and a *narL* knockout mutant revealed the different synthesis of seven proteins (Quäck, 2005). Interestingly, synthesis of the arginine fermentation proteins ArcABC was found increased in the *narL* mutant compared to the wild type implying a repression of the corresponding genes by NarL. In *E. coli*, NarL was found to repress transcription of genes encoding alternative respiratory systems or genes involved in fermentation (Constantinidou *et al.*, 2006). However, so far no direct repression of genes by the *P. aeruginosa* NarL regulator is described.

Since the *arcDABC* operon was not identified throughout the microarray analysis as shown in tab. 3.2, the impact of NarL on expression of the arginine fermentation genes encoded by the *arcDABC* operon was investigated using northern blot analysis and *lacZ* reporter gene experiments.

3.1.2.1 Northern blot analysis of *arcDABC* mRNA

For northern blot analysis, RNA was isolated from the *P. aeruginosa* wild type PAO1 and the *narL* mutant PAO9104 growing under oxygen limited conditions in LB medium as described for the microarrays. RNA preparation, agarose gel electrophoresis and northern blotting were carried out as described in Materials and Methods. Northern blot analysis using 10 µg of total RNA hybridized with a DIG-labeled *arcA* probe revealed the typical *arcABC, arcAB* and *arcA* transcripts under the tested conditions, however the *arcDABC* transcript was not detected as shown in fig. 3.4 (Gamper *et al.*, 1992).

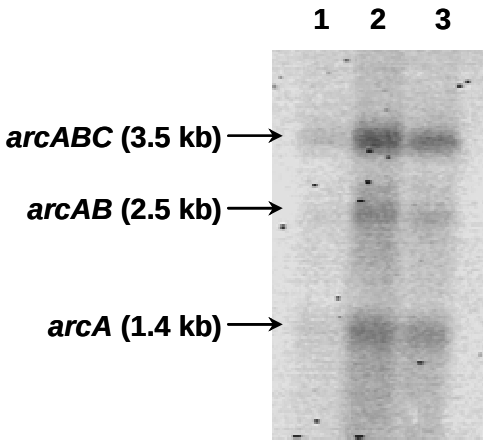


Figure 3.4: Northern blot analysis of *arcDABC* mRNA from (Benkert *et al.*, 2008). RNA was prepared from the wild type grown under oxygen limitation in LB medium with 50 mM KNO_3 (lane 1), the wild type grown without KNO_3 (lane 2) and the *narL* mutant incubated in LB medium supplemented with 50 mM KNO_3 (lane 3). Total RNA was isolated after 3 h incubation at 37℃. The mRNAs were detected using an *arcA* specific probe (Trunk, 2005).

By comparing mRNA levels from the wild type grown with 50 mM KNO_3 (lane 1) and without nitrate (lane 2) strong signals were only detected for the wild type grown without nitrate confirming that the presence of nitrate resulted in decreased *arcDABC* transcription. Most importantly, mRNA levels from the *narL* mutant grown with nitrate (lane 3) resulted in almost identical signal intensities compared to the wild type incubated without the addition of nitrate (lane 2) clearly indicating the NarL-dependent repression of *arcDABC* expression.

3.1.2.2 The *arcDABC* operon is repressed by NarL

Transcriptional promoter *lacZ* reporter gene fusions were used to test the influence of NarL on the *arcDABC* promoter. Therefore, the complete *arcD* promoter region was fused to *E. coli lacZ* and integrated into the *attB* site of the *P. aeruginosa* genome as described in Materials and Methods. β-Galactosidase activities of P_{arcD}-*lacZ* carrying strains BB43 (wild type) and BB45 (*narL* mutant) were determined under anaerobic denitrifying conditions as described for the proteome analysis (Quäck, 2005). Promoter activity increased more than threefold in the *narL* mutant (BB45, 1975 Miller Units MU) compared to the wild type (BB43, 550 MU) suggesting that the absence of NarL leads to an increased transcription of *arcD*. Consequently, NarL is responsible for *arcDABC* repression.

3.1.2.3 Identification of a NarL binding site in the *arcDABC* promoter

Previous studies showed, that expression of the *arcDABC* operon depends on the global oxygen sensing regulator Anr and the arginine responsive regulator ArgR (Gamper *et al.*, 1991; Lu *et al.*, 1999). An Anr binding site at - 41.5 nucleotides (nt) and an ArgR binding site spanning from - 94 to - 53 nt relative to the transcriptional start site could be functionally identified in the *arcD* promoter region (Gamper *et al.*, 1991; Lu *et al.*, 1999).

To confirm a direct repression of the *arcDABC* operon by NarL, the *arcD* promoter region was searched for the presence of a NarL binding site using the Virtual Footprint tool of the PRODORIC database (Münch *et al.*, 2005). A conserved heptameric NarL binding site was found at - 60 nt of the transcriptional start site in the *arcD* promoter and differs in one position to the published *E. coli* NarL consensus sequence reading $TAC^{C}/_{T}N^{A}/_{C}T$, see fig. 3.5 (Tyson *et al.*, 1993).

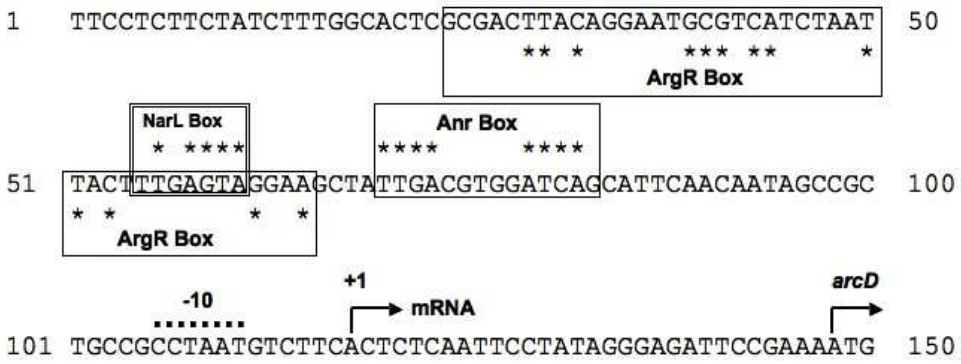


Figure 3.5: Nucleotide sequence of the *arcDABC* promoter region from (Benkert *et al.*, 2008).
The previously determined transcriptional start site, the putative -10 region and the start of the *arcD* coding region are marked (Galimand *et al.*, 1991). Binding sites for the regulatory proteins ArgR, NarL and Anr are boxed. Bases identical to published consensus sequences are marked by an asterisk (Galimand *et al.*, 1991; Lu *et al.*, 1999; Spiro and Guest, 1990). Compared to the ArgR box, the orientation of the NarL binding site is in opposite direction to the transcriptional start site (TACTCAA).

The position of the putative NarL binding site overlaps with the published ArgR binding site suggesting that NarL interferes with ArgR, but not with Anr. In order to functionally confirm the bioinformatics prediction, the putative NarL binding site in the *arcD* promoter was mutated (TACTCAA→CATTCAA) based on the published NarL consensus sequence from *E. coli* using the Cross-over PCR technique as described in Materials and Methods (Ho *et al.*, 1989; Tyson *et al.*, 1993). β-Galactosidase activities from strain BB46 containing the mutated NarL binding site (1696 MU) were similar to those obtained for the *narL* mutant strain BB45 with the wild type promoter (1975 MU). This result confirms a direct repression of the *arcDABC* promoter by NarL.

3.1.2.4 Arginine-dependent ArgR activation of *arcDABC* is repressed by nitrate-dependent NarL

For elucidation whether NarL interferes with the Anr or ArgR regulator, promoter *lacZ* reporter gene experiments were extended for the separate addition of nitrate and arginine to defined AB minimal medium.

Highest β-galactosidase activities of P_{arcD}-*lacZ* in the wild type (BB43) and the *narL* mutant (BB45) were obtained for anaerobic conditions in the presence of 20 mM arginine, see tab. 3.4. Addition of 50 mM nitrate decreased β-galactosidase activity by 55 % in the wild type, but only 24 % in the *narL* mutant stain and 19 % in the wild type strain containing the mutated NarL binding site (BB46). This clearly showed NarL-dependent repression of arginine mediated ArgR activation of the *arcD* promoter via the proposed and identified NarL binding site.

Table 3.4: Expression of the P_{arcD} -*lacZ* reporter gene fusions in the *P. aeruginosa* wild type and the *narL* mutant.

***P. aeruginosa* strain**	***lacZ* fusion**	**Strain background**	**β-Galactosidase activity***			
			-	**- + NO_3^-**	**+ Arg -**	**+ Arg + NO_3^-**
BB43	P_{arcD}	PAO1 (wt)	103	92	621	275
BB45	P_{arcD}	PAO9104 (*narL*$^-$)	233	305	666	505
BB46	$P_{arcD\Delta NarL}$	PAO1 (wt)	469	431	2364	1916

* β-Galactosidase activities of the *lacZ* reporter gene fusions are given in Miller units. Bacteria were grown under oxygen limited conditions in modified AB minimal medium. As indicated, 50 mM KNO_3 and 20 mM arginine (Arg) were added. After seven hours incubation at 37 ℃ samples were taken for the β-galactosidase assay. β-Galactosidase activities are the mean of at least three independent experiments. Standard deviations ranged between 3 to 15 %.

However, NarL does not completely abolish expression of the *arcDABC* operon in the presence of nitrate (fig. 3.4 and tab. 3.4). In the presence of nitrate NarL prevents additional activation of *arcDABC* via ArgR, but not the basic expression mediated by the Anr regulator. This is in agreement with recently published data, where the authors observed expression of the *arcDABC* operon under anaerobic denitrifying conditions in complex medium containing arginine (Platt *et al.*, 2008). Moreover, expression of the *arcDABC* operon was found increased in the presence of nitrite compared to nitrate (Platt *et al.*, 2008). Thus, in the presence of nitrate and arginine, NarL binding most likely prevents interaction of the arginine-dependent ArgR activator with its overlapping binding site and represses further induction of *arcDABC* transcription by ArgR. Increased β-galactosidase activity of the P_{arcD}-*lacZ* fusion in the *narL* mutant strain under anaerobic conditions without the addition of nitrate and arginine indicates that NarL represses *arcDABC* transcription also in the absence of arginine, see tab. 3.4.

Results of the *lacZ* reporter gene fusion experiments as well as the position of the functionally identified NarL binding site suggest the following model:

Under anaerobic denitrifying conditions the NarX-NarL regulatory system of *P. aeruginosa* is employed for activation of nitrate respiration and downregulation of arginine fermentation demonstrating the concept of a double role for NarL as an activator for nitrate reductase formation as well as a repressor of energetically less effective fermentative pathways, see fig. 3.6.

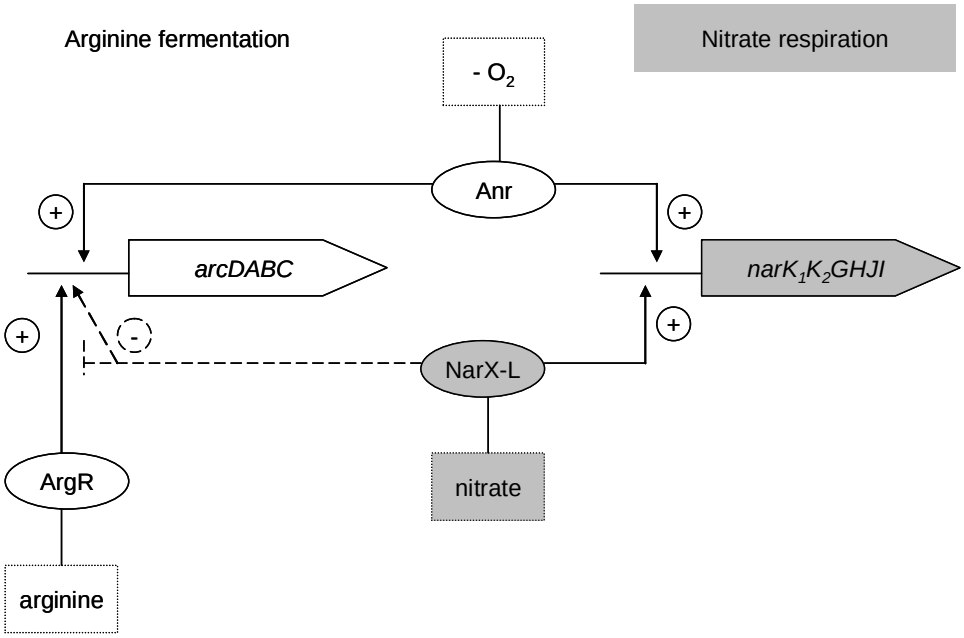


Figure 3.6: Schematic representation of NarX-NarL-dependent regulation from (Benkert *et al.*, 2008).

The major regulator for anaerobic growth is Anr, which activates *arcDABC* even in the absence of arginine and nitrate. In the presence of arginine the ArgR regulator positively stimulates transcription of the *arcDABC* operon. If arginine and nitrate are available, the NarL regulator represses the ArgR-dependent *arcDABC* stimulation by binding to the corresponding promoter region. On the other hand, expression of the nitrate reductase operon *narK$_1$K$_2$GHJI*, which catalyzes the first step in denitrification, is induced by the nitrate response system NarX-NarL.

3.1.3 Two redox regulators and one DNA binding site: essentials for specific gene regulation by Anr and Dnr in *Pseudomonas aeruginosa*

Previous work in our laboratory showed, that the *P. aeruginosa* Anr regulon contains approximately 122 members as determined by an iterative transcriptome, proteome and bioinformatics approach (Trunk, unpublished data). The Dnr regulon consists of only a few members, all devoted to denitrification. A bioinformatics analysis was used for detection of Anr- or Dnr specific determinants leading to differentiation into three functional groups of Anr boxes. Interestingly, all four promoters which are solely Dnr-dependent as well as the three Dnr- and Anr-dependent promoters contained exclusively highly conserved group I Anr boxes. Additionally, group I also contains 12 solely Anr-dependent promoters assuming that the palindromic core sequence does not provide the sole structural basis for the Anr or Dnr specificity of target promoters and that additional elements might be localized in the up- and downstream promoter regions of the Anr-box. A bioinformatics approach searching the up- and downstream regions of all solely Anr- and Dnr-dependent promoters for specific elements failed to detect conserved DNA sequences in these regions. Therefore, the problem was approached experimentally by using *lacZ* reporter gene experiments. For this purpose the experimentally verified Anr-dependent *arcD* and the Dnr-dependent *nirS* promoter were selected.

3.1.3.1 Contribution of promoter elements to Anr and Dnr specific gene regulation in *Pseudomonas aeruginosa*

To investigate the impact of different promoter regions on Anr- or Dnr dependence, two regions in both promoters were defined. First, the corresponding downstream region (ds), which starts at the 3'-end of the respective Anr and Dnr binding sites and ends at the start codon of the respective gene. This downstream region contains the binding site of the sigma factor (- 10 region). Second, the upstream region (us) spans over 300 bp region at the 5'-end of the respective Anr and Dnr binding sites and may contain contact regions for the alpha subunit of the RNA-polymerase and for additional transcription factors. All possible combinations of these elements were cloned and fused to *lacZ* as reporter gene as described in Materials and Methods. β-Galactosidase activities of various promoter constructs were first determined under anaerobic denitrifying conditions, see fig. 3.7.

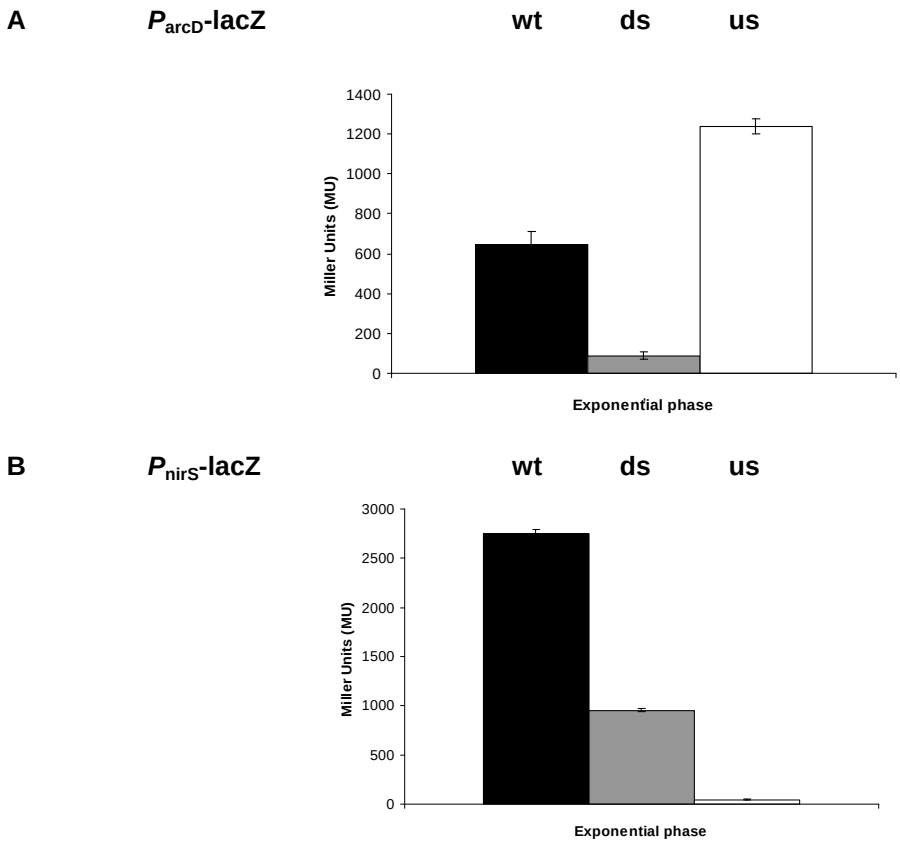


Figure 3.7: Expression of the P_{arcD} -*lacZ* and P_{nirS} -*lacZ* reporter gene fusions in the *P. aeruginosa* wild type PAO1.

β-Galactosidase activities of the *lacZ* reporter gene fusions are given in Miller units. Bacteria were grown under anaerobic conditions in LB medium supplemented with 50 mM KNO_3 at 37 ℃. After 4 h hours samples were taken for enzyme assay. β-Galactosidase activities are the mean of at least three independent experiments. Standard deviations ranged between 3 to 15 %. Black bars: wild type promoter; grey: promoter with the exchanged downstream region (ds); white: promoter with the exchanged upstream region (us).

Exchange of the downstream region dramatically decreased β-galactosidase activity of the *arcD* promoter, whereas *nirS* activity decreased only twofold indicating the importance of a correct downstream region, which consists of the - 10 region, of Anr-dependent promoters. In contrast to the *arcD* promoter showing highest β-galactosidase activity, exchange of the upstream region resulted in loss of *nirS* promoter activity implying that the upstream region is required for Dnr but not for Anr specificity.

In order to check the diverse promoter constructs for Anr- and Dnr-dependence, β-galactosidase activities of the promoter *lacZ* fusion were measured in the *P.*

aeruginosa wild type and the *anr* and *dnr* mutant strains. Since both mutant strains did not grow under anaerobic conditions, shift experiments were applied as described elsewhere (Trunk, 2005). Results are given in tab. 3.5.

Table 3.5: Expression of various *P-lacZ* reporter gene fusions in *P. aeruginosa* wild type PAO1, Δ*anr* mutant and Δ*dnr* mutant.

	[b] Promoter construct			[a] β-Galactosidase activity (Miller units)			
				PAO1	Δ*anr*	Δ*dnr*	Regulator
1	us	Anr	ds	1046	104	463	Anr
2	us	Anr	ds	823	37	287	Anr
3	us	Dnr	ds	734	48	210	Anr
4	us	Dnr	ds	1229	20	90	Dnr
5	us	Anr	ds	105	9	35	Dnr
6	us	Dnr	ds	54	11	11	Dnr

[a] β-Galactosidase activities of the promoter-*lacZ* reporter gene fusions in the wild type PAO1, the *anr* mutant PAO6261 and the *dnr* mutant BB71 are given in Miller units. *P. aeruginosa* cells were grown aerobically to an OD_{578} of 0.3 and then shifted to anaerobic flasks. Four hours after the shift, samples were taken for the β-galactosidase assay.

[b] Schematic representation of the *arcD* and *nirS* promoter regions. "us" refers to the sequence upstream of the Anr box. "Anr" or "Dnr" refer to the Anr box in the *arcD* or *nirS* promoter region, respectively. "ds" denotes the promoter region downstream of the Anr box. White boxes indicate promoter sequences of the *arcD*, grey boxes indicate promoter sequences of the *nirS* promoter.

Since the *arcD* promoter with the exchanged downstream region showed very low β-galactosidase activity, various hybrid promoters all containing the *arcD* downstream region (dsAnr) were first analyzed (tab. 3.5, no. 1 to 3). Interestingly, all three promoter constructs were highly active under anaerobic denitrifying conditions, even when the *arcD* downstream region (dsAnr) was combined with the Dnr binding site and the upstream region (usDnr) of the *nirS* promoter (no. 3). Most importantly, promoter constructs no. 1 - 3 revealed Anr-dependent expression irrespective of the central Dnr binding site or the upstream region (usDnr) of the *nirS* promoter. Moreover, the wild type *arcD* promoter (no. 1) and hybrid promoter no. 2 showed the highest β-galactosidase activities. Both shared a central Anr binding site and the downstream region of *arcD* (dsAnr). However, promoter activity of hybrid promoter no. 3 is similar to no. 2 sustaining the assumption from the bioinformatics analysis

that Anr and Dnr specificity is not only mediated via the binding site. Apparently, the *arcD* Anr box downstream region determined Anr-dependency for those constructs.
Next, the hybrid promoters all sharing the *nirS* downstream region (dsDnr) were investigated. Surprisingly, β-galactosidase activities of the two hybrid promoters no. 5 and 6 containing the upstream promoter region of the *arcD* Anr box (usAnr) dramatically decreased under anaerobic denitrifying conditions. Consequently, the *nirS* Dnr binding site downstream promoter region (dsDnr) is not compatible to *arcD* Anr box upstream region (usAnr). Dnr-dependence with efficient transcription was only observed for the wild type *nirS* promoter (no. 4). Nevertheless, decreased promoter activity of constructs no. 5 and 6 in both mutant strains compared to the wild type implied Dnr-dependent regulation. Obviously, an efficient Dnr-dependent promoter requires both a highly conserved Dnr binding site and the *nirS* up- and downstream regions. It could also be speculated that another regulator is involved in Dnr specificity by binding to an element located in the upstream region.
Obtained results from the promoter *lacZ* studies using hybrid *arcD* and *nirS* promoters clearly demonstrated, that the promoter region downstream of the Anr box of an Anr- and Dnr-dependent promoter mediates Anr- and Dnr specificity and that Dnr specificity further requires the corresponding upstream region.

3.2 ANALYSIS OF *PSEUDOMONAS AERUGINOSA* BY GC-MS BASED METABOLOMICS

Metabolomics is a relatively new field in sciences and thus only few publications are available mostly focusing on establishment of the method and technique used for metabolome analysis (Jaki *et al.*, 2006; Maharjan and Ferenci, 2003; Strelkov *et al.*, 2004; Villas-Boas *et al.*, 2005). Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) are commonly used techniques for investigations of an organisms metabolome (Rochfort, 2005). Recently, NMR metabolomics was applied for analysis of *P. aeruginosa* biofilms and planktonic cultures simply showing the distinct nature of these two modes of growth (Gjersing *et al.*, 2007). A more detailed approach using liquid chromatography - mass spectrometry (LC-MS) revealed first insights into metabolite composition of *Pseudomonas putida* cells grown on different carbon sources (van der Werf *et al.*, 2008).

For comprehensive analysis of the *P. aeruginosa* metabolome, high-throughput metabolomics based on gas chromatography - mass spectrometry (GC-MS) was established in the beginning of this work. A method for sample preparation, metabolite derivatization and GC-MS measurement, previously developed for *Corynebacterium glutamicum,* was optimized for *P. aeruginosa* and reproducibility was confirmed (Strelkov *et al.*, 2004; Thielen, 2008). In a first approach, *P. aeruginosa* cells grown planktonically and as biofilms were investigated. Clearly, different metabolic profiles were demonstrated for these two conditions (Klawonn, 2007). Moreover, the method was successfully applied for characterization of phenotypes by the use of mutants identifying a lysine decarboxylase, encoded by PA1818, in *P. aeruginosa* (Thielen, 2008).

In the second part of this work, GC-MS based metabolomics was used for investigation of growth phase related changes of *P. aeruginosa* during aerobic life cycle. Additionally, microarray analyses were performed and finally used for combination of transcriptome and metabolome data in order to study the cellular response of *P. aeruginosa* on the transcriptional and metabolic level.

3.2.1 Determination of growth phase related changes of *Pseudomonas aeruginosa* using metabolomics in combination with transcriptomics

For a systematic investigation of the *P. aeruginosa* metabolism during adaptation from exponential to stationary growth phase, a comprehensive approach using the Affymetrix *Pseudomonas* GeneChips® and the established GC-MS technique was applied. Both high-throughput data sets were combined for providing a first global picture of the bacterium to changing growth conditions on transcriptional and metabolic level.

3.2.1.1 Experimental set up for metabolome and transcriptome analyses

P. aeruginosa PAO1 wild type cells were grown under aerobic conditions in AB minimal medium supplemented with 20 mM glucose as sole carbon source. Samples for GC-MS and microarray experiments were collected at four different time points representing exponential growth phase (4 h; OD_{578} ~ 0.7), transition phase between exponential and stationary phase (7 h; OD_{578} ~ 3.0), early stationary phase (10 h; OD_{578} ~ 3.5) and late stationary phase of growth (13 h; OD_{578} ~ 3.4), see fig. 3.8.

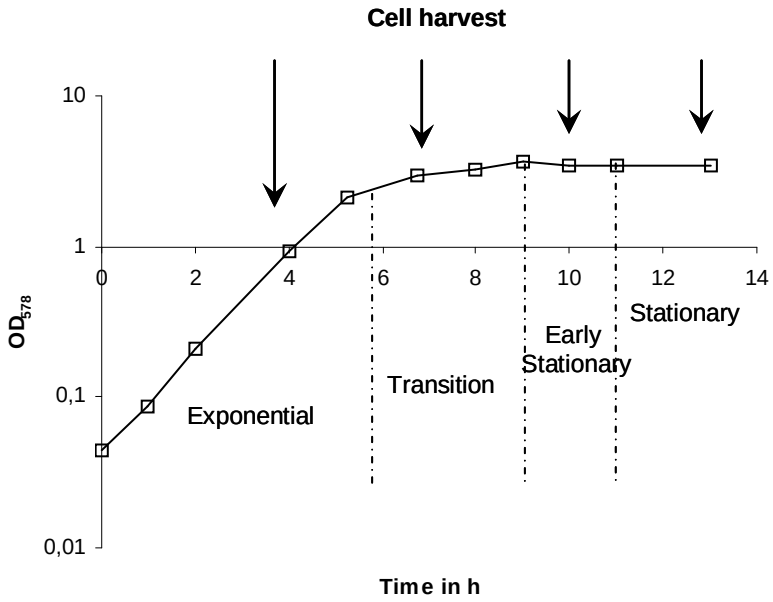


Figure 3.8: Growth curve of *P. aeruginosa* under aerobic conditions.

P. aeruginosa PAO1 wild type cells were grown under aerobic conditions in AB minimal medium supplemented with 20 mM glucose at 37 °C. Four different growth modes were defined for cell harvest as indicated by an arrow: exponential phase, transition phase, early stationary phase and stationary phase.

Sample preparation, metabolite derivatization, GC-MS measurement and data processing were carried out as described in Materials and Methods and elsewhere (Strelkov *et al.*, 2004; Thielen, 2008). Five biological replicates were used for GC-MS analysis. For statistical analysis of metabolome data a likelihood ratio test was applied showing significant differences (P-value < 0.01) between two conditions (Klawonn, 2007).

RNA extraction, cDNA synthesis, fragmentation and final labeling were performed as described in Materials and Methods. Three biological and three technical replicates were used for microarray analysis. Target hybridization, washing, staining and scanning of GeneChips® were performed by the Affymetrix Core Facility at the Helmholtz Centre for Infection Research (HZI), Braunschweig. For analysis of transcriptome data the Bioconductor software package was used as described in Materials and Methods and elsewhere (Bolstad *et al.*, 2003; Gentleman *et al.*, 2004; Irizarry *et al.*, 2003a; Irizarry *et al.*, 2003b).

3.2.1.2 The *Pseudomonas aeruginosa* metabolome

With the help of the employed GC-MS technique, a total of 147 *P. aeruginosa* metabolites with a significant peak area above background were detected, see tab. 3.6. Since the PseudoCyc pathway/genome database for *P. aeruginosa* PAO1 predicts 678 metabolites (Romero and Karp, 2003), this analysis covered over 20 % of these metabolites. While the identity of 44 metabolites is still unknown, 103 metabolites were identified by their retention index and mass spectrum, see tab. 3.6. The number of identified metabolites is in the similar order as found in other microorganisms. The same GC-MS technique led to the identification of 121 metabolites in *C. glutamicum* (Strelkov *et al.*, 2004). Further, over 150 peaks were determined in a GC-MS based metabolomics approach investing the yeast metabolome (Villas-Boas *et al.*, 2005).

Table 3.6: Detected intracellular metabolites of *P. aeruginosa* by GC-MS.

Amino acids & derivates	Organic acids	Others	Fatty acids & derivates	Sugars
alanine	benzoate	adenine	dodecanoic acid	fructose
aspartate	borate	adenosine	hexadecanoic acid	galactose
β-alanine	carbonate	AMP	octadecanoic acid	glucose
cycloleucine	citrate	cadaverine	oleic acid amide	maltose
cysteine	fumarate	cytosine	palmitic acid amide	mannose
glutamate	glutarate	ethanolamine		sucrose
glutamine	glycerate	glyceraldehyde	**Fatty alcohols**	trehalose
glycine	glycerol	hydrogen sulfide	dodecanol	xylose
homoserine	glycerol-3-phosphate	hydroxylamine	hexadecanol	1.6-anhydro-β-glucose
isoleucine	glycerone-phosphate	hypoxanthine	octadecanol	
leucine	glycolate	nicotinamide	tetradecanol	**Sugar phosphates**
lysine	lactate	phosphoethanolamine	1-mono-oleoylglycerol	erythrose-4-phosphate
methionine	malate	putrescine	1-mono-palmitoylglycerol	fructose-6-phosphate
O-phosphoserine	N-acetyl-glutamate	spermidine	1-mono-stearoylglycerol	glucosamine-6-phosphate
ornithine	nicotinate	thymine		glucose-6-phosphate
phenylalanine	phosphate	triethanolamine		mannose-6-phosphate
proline	phosphoenol-pyruvate	uracil		xylulose-5-phosphate
serine	pyrophosphate	urea		
threonine	pyruvate	uridine		**Sugar alcohols**
tryptophane	quinate	UMP		mannitol
tyrosine	succinate	1,3-diamino-propane		ribitol*
valine	succinate methylester	2-hydroxy-pyridine		
4-aminobenzoate	2-ketoglutarate	5'-methyl-thioadenosine		
4-aminobutyrate	2-hydroxybutyrate			
5-aminolevulinate	2-hydroxyglutarate			
5-oxoproline	3-phosphoglycerate			

*Ribitol was used as internal standard.

Most of the identified metabolites are amino acids and their derivates and organic acids, see tab. 3.6. With the exception of arginine and histidine, all proteinogenic amino acids and several amino acid derivates were detected in this experiment via GC-MS. However, while histidine was found in other *P. aeruginosa* metabolome experiments in our laboratory arginine is still undetectable, which might be due to fast reconversion.

Furthermore, intermediates of central pathways as the energy generating Entner-Doudoroff pathway and intermediates of the tricarboxylic acid (TCA) cycle essential for several biosynthetic pathways were identified.
Metabolites grouped as “others” are partly essential components of enzymatic cofactors as well as nucleosides/nucleotides and furthermore, natural polyamines essential for optimal growth and viability were measured.
Fatty acids and their derivates as well as fatty alcohols were also detected in this experiment. In addition, various sugars, sugar phosphates and sugar alcohols were determined by GC-MS analysis. Notably, the group of sugar phosphates included intermediates of the pentose phosphate pathway providing substrates for nucleotide biosynthesis and NADPH.
Altogether, over 100 metabolites involved in several important cellular processes were identified by using this GC-MS based metabolomics approach allowing global analysis of the *P. aeruginosa* metabolome.

3.2.1.3 Metabolic response of *Pseudomonas aeruginosa* to changing growth phases

Metabolome analysis of aerobically grown *P. aeruginosa* cells revealed a total of 58 metabolites showing significant changes in relative concentrations in response to different growth phases, see tab. 3.7. As expected, highest differences of the metabolic patterns were observed when cells from exponential phase were compared with cells from early or late stationary phase. Relative concentrations of more than 40 metabolites were found changed between growing and resting cells. In contrast, relative concentrations of only 17 metabolites changed between exponential and transition phase of growth. The complete list of *P. aeruginosa* metabolites, which significantly changed between two conditions, is given in tab. 3.7. For a better understanding of metabolome data, identified metabolites were classified according to the KEGG database (www.genome.jp/kegg/) to allow global insights into the *P. aeruginosa* metabolism during aerobic life cycle.

Table 3.7: Metabolic changes of *P. aeruginosa* in response to different growth phases.

Metabolite[a]	Pathway[b]	Ratio[c] Tr / Ex	ESt / Ex	St / Ex
UMP	Glycan biosynthesis and metabolism Nucleotide metabolism	0.16	2.54	4.19
citrate	Carbohydrate metabolism Amino acid metabolism Signal transduction	0.17	0.10	0.08
2-hydroxybutyrate	Carbohydrate metabolism	0.17	0.25	0.16
O-phosphoserine	Amino acid metabolism	0.18	0.08	0.04
glucosamine-6-phosphate	Amino acid metabolism Carbohydrate metabolism Membrane transport	0.22	0.14	0.01
5-aminolevulinate	Amino acid metabolism Metabolism of cofactors and vitamins	0.24	0.14	0.05
glutamine	Amino acid metabolism Metabolism of other amino acids Nucleotide metabolism Energy metabolism Membrane transport Translation	0.31	0.09	0.02
lactate	Carbohydrate metabolism	0.40	0.35	0.12
2-hydroxyglutarate	Carbohydrate metabolism		0.07	0.06
N-acetyl-glutamate	Amino acid metabolism		0.13	0.03
fructose-6-phosphate	Carbohydrate metabolism		0.25	0.21
alanine	Amino acid metabolism Metabolism of other amino acids Translation		0.28	0.16
phenylalanine	Amino acid metabolism Translation		0.43	0.32
isoleucine	Amino acid metabolism Membrane transport Translation			0.14
valine	Amino acid metabolism Carbohydrate metabolism Membrane transport Translation Metabolism of cofactors and vitamins			0.15
leucine	Amino acid metabolism Membrane transport Translation			0.15
mannitol	Carbohydrate metabolism Membrane transport			0.16
β-alanine	Amino acid metabolism Carbohydrate metabolism Metabolism of other amino acids Metabolism of cofactors and vitamins Nucleotide metabolism			0.20
malate	Carbohydrate metabolism Amino acid metabolism			0.29
xylulose-5-phosphate	Carbohydrate metabolism			0.33
aspartate	Amino acid metabolism Metabolism of other amino acids Metabolism of cofactors and vitamins Energy metabolism Membrane transport Translation Cell motility			0.38

Table 3.7 continued

Metabolite[a]	Pathway[b]	Ratio[c] Tr / Ex	ESt / Ex	St / Ex
serine	Amino acid metabolism Metabolism of other amino acids Energy metabolism Translation			0.38
hypoxanthine	Nucleotide metabolism	42.01	31.87	16.32
cycloleucine	No information available	21.94	34.54	22.54
ornithine	Amino acid metabolism Metabolism of other amino acids Membrane transport	16.18	20.81	39.33
oleic acid amide	Lipid metabolism	8.34		
ethanolamine	Lipid metabolism Metabolism of other amino acids	7.29	41.85	40.80
palmitic acid amide	Lipid metabolism	4.75	7.52	
1,6-anhydro-beta-D-glucose	No information available	3.24	10.21	5.52
nicotinamide	Metabolism of cofactors and vitamins	3.15	5.26	5.32
xylose	Carbohydrate metabolism Membrane transport	2.43	2.53	
glutarate	Amino acid metabolism Lipid metabolism		9.31	16.96
maltose	Carbohydrate metabolism Membrane transport Cell motility		5.95	5.59
nicotinate	Metabolism of cofactors and vitamins		4.49	5.08
octodecanol	Lipid metabolism		4.22	
lysine	Amino acid metabolism Metabolism of cofactors and vitamins Membrane transport Translation		4.09	4.59
quinate	Carbohydrate metabolism		3.92	5.10
methionine	Amino acid metabolism Translation		3.56	
hexadecanol	Lipid metabolism		3.56	
1-monostearoylglycerol	Lipid metabolism		3.24	
tetradecanol	Lipid metabolism		3.21	
pyrophosphate	Energy metabolism		3.21	4.66
1-monopalmitoylglycerol	Lipid metabolism		3.12	
hydroxylamine	Energy metabolism		3.01	
tyrosine	Amino acid metabolism Translation Metabolism of cofactors and vitamins		2.98	
adenine	Nucleotide metabolism Amino acid metabolism		2.79	2.66
cysteine	Amino acid metabolism Energy metabolism Metabolism of cofactors and vitamins Translation		2.76	
mannose	Carbohydrate metabolism Membrane transport		2.75	
hexadecanoic acid	Lipid metabolism		2.39	
AMP	Nucleotide metabolism		2.25	4.74
cadaverine	Amino acid metabolism Metabolism of other amino acids			180.16
triethanolamine	Lipid metabolism			12.35
thymine	Nucleotide metabolism			5.27
adenosine	Nucleotide metabolism			4.14

Table 3.7 continued

Metabolite[a]	Pathway[b]	Ratio[c] Tr / Ex	ESt / Ex	St / Ex
sucrose	Carbohydrate metabolism Membrane transport			4.08
uridine	Nucleotide metabolism			3.64
uracil	Nucleotide metabolism Metabolism of other amino acids			2.52

[a] *P. aeruginosa* metabolites showing significant differences between two conditions.

[b] Pathways are according to the KEGG database (www.genome.jp/kegg/).

[c] Ratio means fold change in relative metabolite concentrations between two conditions of at least < 0.5 and > 2. Ex: exponential phase; Tr: transition phase; ESt: early stationary phase; St: stationary phase.

As shown in tab. 3.7, levels of nearly all metabolites found differentially produced in exponential phase compared to transition phase, with exception of UMP, changed over time of incubation indicating down- or upregulation of corresponding pathways in response to different growth phases. As expected, intermediates of central energy generating pathways essential for growth of bacteria were found in lower concentrations in transition and stationary phase than in exponential phase, while low-energy indicating metabolites such as AMP and UMP were found increased in stationary phase.

Relative concentrations of two intermediates of the tricarboxylic acid cycle (TCA cycle) were found decreased during stationary phase indicating downregulation of this central pathway in resting cells. Whereas levels of citrate, an intermediate of the first half of TCA cycle, decreased within transition and stationary phase, amounts of malate, an intermediate of the second half of TCA cycle were found decreased not until later stationary phase.

Moreover, two intermediates of the pentose phosphate pathway providing substrates for nucleotide biosynthesis and NADPH production, fructose-6-phosphate and xylulose-5-phosphate, were detected. Relative concentrations of both phosphorylated sugars were additionally found in lower concentrations in stationary phase indicating downregulation of the respective pathways. While levels of fructose-6-phosphate, which can also be used as substrate for amino sugar synthesis, were found decreased during early and later stationary phase, relative concentrations of xylulose-5-phosphate were found reduced during later stationary phase.

Amounts of O-phosphoserine involved in biosynthesis of serine, cysteine and glycine were found decreased upon entry into transition phase and in stationary phase. No

changes of these amino acids itself were detected between transition and exponential phase, but serine levels decreased in stationary phase and cysteine was found increased in early stationary phase. Reduced concentrations of glucosamine-6-phosphate and lactate were also found in stationary phase compared to exponential phase. Note that all these metabolites were synthesized from intermediates of glycolysis, but however, no changes of corresponding intermediates were observed.

Further, levels of 5-aminolevulinate, the general precursor of heme biosynthesis, decreased upon entry into transition phase and stationary phase. Hemes are essential cofactors of proteins involved in both aerobic and anaerobic respiratory chains. Downregulation of heme biosynthesis is in accordance with slower growth and indicates oxygen limitation during transition and stationary phase. Consistent with oxygen limiting conditions and the absence of an alternative electron acceptor, amounts of ornithine were found increased during transition and stationary phase. Ornithine is produced via arginine fermentation, which is used in *P. aeruginosa* for energy production under oxygen limited conditions in the absence of alternative electron acceptors (Vander Wauven *et al.*, 1984).

Growth phase specific changes in relative metabolite concentrations were additionally observed for amino acids mostly found downregulated in stationary cells. Glutamine levels already decreased during transition phase, which might be due to involvement in several metabolic pathways. Among others, glutamine provides nitrogen for purine and pyrimidine biosynthesis and additionally for amino sugar production, which is consistent with decreasing amounts glucosamine-6-phosphate in transition and stationary phase.

Upon entry into early stationary phase, decreasing amounts of another two amino acids, alanine and phenylalanine, were detected. Significantly lower concentrations of valine, leucine, isoleucine, serine and aspartate were first noted in late stationary phase of growth suggesting overall downregulation of amino acid biosynthesis and thus protein biosynthesis in stationary cells.

Unexpectedly, three amino acids, cysteine, methionine and tyrosine, were found in high amounts during early stationary phase. Since methionine provides sulfur for cysteine, cysteine biosynthesis is connected to methionine metabolism explaining high levels of both amino acids. Accumulation of cysteine might be due to higher demand of sulfur essential for biosynthesis of iron-sulfur cluster containing proteins

under low oxygen conditions. On the other hand, methionine metabolism is linked to N-acyl-homoserine lactone (AHL) biosynthesis. AHLs are used as signal molecules in QS influencing virulence factor production and biofilm formation in a cell density dependent manner reviewed in (Venturi, 2006). Tyrosine is produced via degradation of phenylalanine and can be further converted to acetoacetate and fumarate in *P. putida* (Arias-Barrau *et al.*, 2004).

In addition, amounts of lysine were surprisingly found increased during stationary phase. In this context, cadaverine, which is synthesized by degradation of lysine, was found in very high amounts in stationary phase implying an important role in resting cells. Interestingly, cadaverine is a polyamine involved in antibiotic resistance and susceptibility of *P. aeruginosa* and *E. coli* (Kwon and Lu, 2006a, 2006b; Tkachenko *et al.*, 2006). In *E. coli* this polyamine is produced in response to low pH conditions (Meng and Bennett, 1992). However, no changes in pH value were observed in this experiment (data not shown).

Obviously, metabolome analyses of adapting and resting cells of the transition and stationary phase revealed a number of degradation products presumably accumulated due to energy starvation. In agreement, high amounts of AMP and UMP detected in stationary phase clearly showed energy starvation in resting cells. Further, increasing amounts of nicotinamide, an essential component of the enzymatic cofactors NAD/NADP, were found in cells of transition and stationary phase.

Relative concentrations of two fatty acid derivates, palmitic acid amide and oleic acid amide, and palmitic acid (hexadecanoic acid) increased within transition phase and stationary phase. Fatty acids are components of complex lipids like acylglycerines and phosphoglycerides and can be further oxidized via β-oxidation. Interestingly, *P. aeruginosa* enzymes catalyzing fatty acid degradation were found induced *in vivo* during lung infection in cystic fibrosis patients (Kang *et al.*, 2008; Son *et al.*, 2007). In addition, high amounts of monoacylglycerines as well as fatty alcohols and ethanolamine were also measured in the early stationary phase. All together, this indicated an ongoing degradation of membrane lipids probably for energy production in adapting and resting cells.

Curiously, amounts of glucose, the used carbon source in this study, were found higher in resting cells. Accumulation of glucose might be a result of other limited factors essential for growth in stationary phase such as oxygen. Regeneration of

glucose via gluconeogensis seems unlikely, since no changes of other metabolites involved in this pathway were detected. Another possibility is that the increase of glucose levels was due to degradation of the cells LPS layer. In this context, growth phase associated changes of mannose concentrations were observed. However, maltose, sucrose and xylose concentrations were additionally found increased during stationary phase. Whereas levels of xylose increased during transition and early stationary phase, glucose and maltose concentrations were found in higher concentrations in both, early and late stationary phase of growth. Mannose and sucrose were already found increased in early and late stationary phase, respectively. Altogether, accumulation of mono- and disaccharides indicated degradation of polysaccharides in transition and stationary phase.

Levels of hypoxanthine were additionally found increased in transition and stationary phase. In this context, high amounts of adenine and adenosine were found during early and late and late stationary phase, respectively. Adenosine and hypoxanthine are intermediates of adenine degradation and most importantly all metabolites are by-products of AHL biosynthesis in *P. aeruginosa* (Heurlier *et al.*, 2006). Consistent with increasing amounts of methionine in early stationary phase, which is additionally connected to AHL metabolism, this indicated AHL production and thus quorum sensing upon entry into transition phase and stationary phase. On the other hand, accumulation of adenine, adenosine and hypoxanthine might be due do degradation of nucleic acids. In this context, high concentrations of uracil, uridine and thymine were detected in late stationary phase. Note, that purines and pyrimidines can be recycled via nucleotide salvage pathway and reused for new nucleotides saving energy. Such processes might be important for surviving stress periods like stationary phase.

A schematic overview of metabolic changes between different growth phases of the *P. aeruginosa* amino acid and carbohydrate metabolism is given in fig. 3.9.

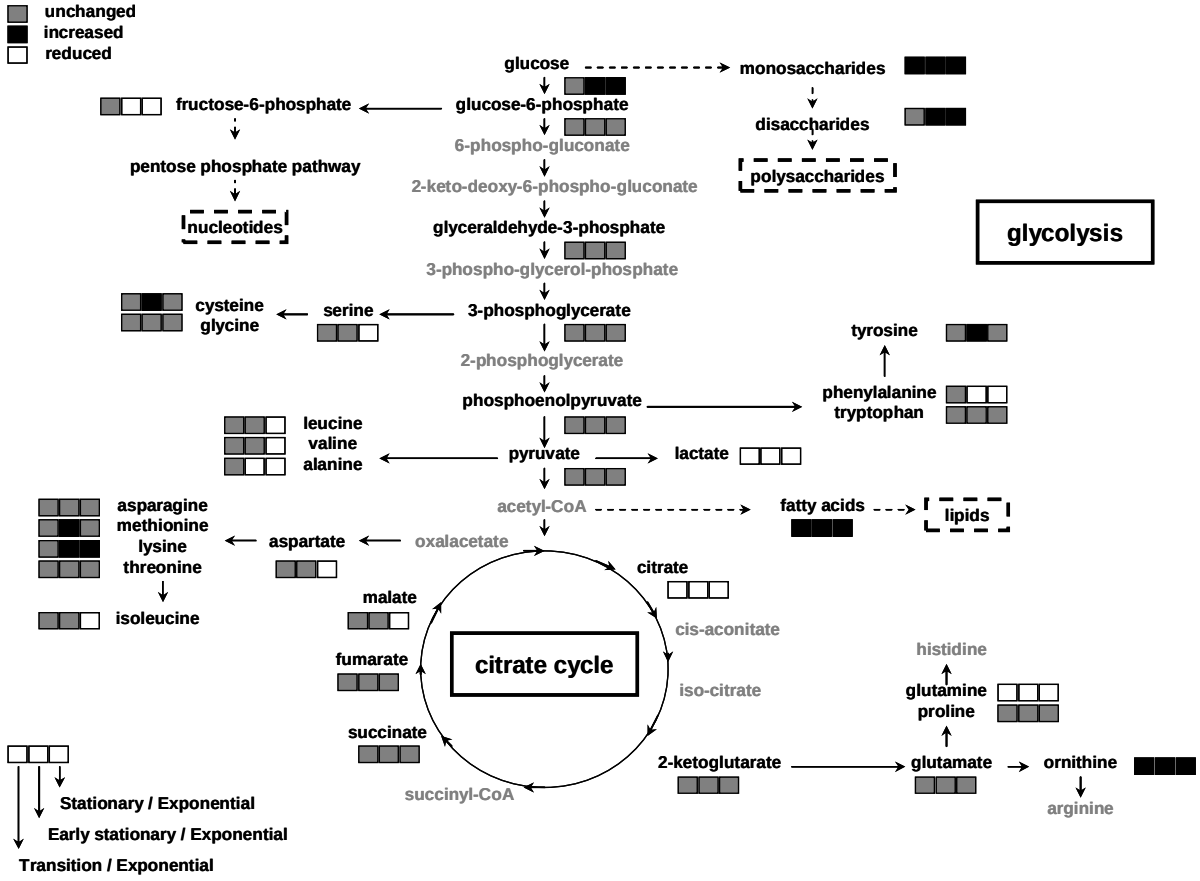


Figure 3.9: Schematic representation of growth phase related metabolic changes of the *P. aeruginosa* metabolism.

Most of the metabolites involved in central metabolic pathways of *P. aeruginosa* were identified by GC-MS analysis and are colored in black. Metabolites which could not be detected in this experiment are colored in grey. Cells of the transition phase, early stationary phase and stationary phase were compared with cells of the exponential phase. Grey boxes: unchanged; black boxes: increased and white boxes: reduced in the corresponding growth phase.

In summary, investigations of growth phase related changes by GC-MS analyses showed overall downregulation of metabolites involved in central pathways including citrate cycle and pentose phosphate pathway in transition and stationary phase, see fig. 3.9. However, no changes of intermediates of the Entner-Doudoroff pathway and glycolysis were observed in this experiment, which might be due to fast conversion of these central metabolites. Consistent with growth phase dependent nutrient and energy availability, activity of such processes is much higher in growing cells than in resting cells. In addition, biosynthesis of most amino acids was found decreased in transition and stationary phase, see fig. 3.9.

Metabolome analyses further demonstrated upregulation of pathways involved in degradation of amino acids, lipids, nucleotides and polysaccharides in adapting and resting cells. Interestingly, detection of growth phase specific amino acids cysteine, methionine and lysine as well as the polyamine cadaverine, which has previously not been described, revealed new informations about metabolic processes in stationary phase which might be essential for surviving various stress conditions and most importantly for adaptation of *P. aeruginosa* during infection.

3.2.1.4 Transcriptional response of *Pseudomonas aeruginosa* to changing growth phases

In agreement with the metabolome analyses, microarrays revealed highest differences in gene expression when comparing exponential and stationary phase of growth. A total of 1418 genes were found differentially expressed between growing and resting cells representing 25 % of the *P. aeruginosa* genome see fig. 3.10. Comparison of exponential and early stationary phase displayed differential expression of 1309 genes and thus 23 % of the genome. Only 10 % (582 genes) were found differentially expressed between exponential phase and transition phase.

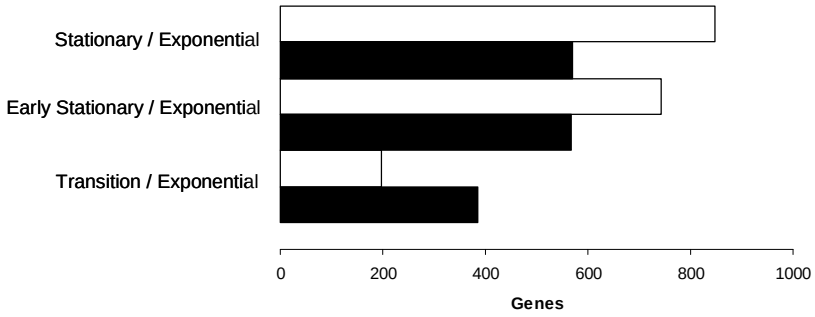


Figure 3.10: Differential gene expression of *P. aeruginosa* in response to changing growth conditions.

For determination of growth phase related changes of the *P. aeruginosa* transcriptome, cells of transition phase, early stationary phase and stationary phase were compared with cells from the exponential growth phase, respectively. Black: induced; white: repressed.

By comparing transition and exponential phase, more genes were found induced in transition phase, see fig. 3.10. In contrast, comparison of early stationary phase and

late stationary with exponential phase, respectively, showed that the majority of genes were repressed in stationary phase.

Genes were assigned to metabolic pathways according to the KEGG database as done for the identified metabolites (www.genome.jp/kegg/). Hypothetical genes and genes which do not encode enzymes allocated to a metabolic pathway were excluded for easier interpretation of microarray data and for comparison with metabolome data. Finally, functional classification led to the identification of 273 genes differentially expressed between transition and exponential phase, see tab. 3.8. In addition, differential transcription of 739 genes and 798 genes was determined by comparison of early stationary phase and later stationary phase with exponential growth phase, respectively. A list of all genes which could be classified is given in Appendix.

Table 3.8: Gene expression of *P. aeruginosa* in response to different growth phases.

Metabolic Pathway	Transition vs. Exponential		Early Stationary vs. Exponential		Stationary vs. Exponential	
Metabolism	+	-	+	-	+	-
Amino acid metabolism	11	19	25	86	22	90
Carbohydrate metabolism	11	13	16	58	17	66
Energy metabolism	28	4	20	54	18	58
Glycan biosynthesis and metabolism	2	2	2	28	3	31
Lipid metabolism	7	7	9	20	6	21
Metabolism of cofactors and vitamins	9	6	1	49	4	51
Metabolism of other amino acids	5	2	1	11	2	12
Nucleotide metabolism	1	3	6	43	7	43
Genetic information processing						
Transcription	11	7	18	19	18	25
Translation	3	41	11	100	11	100
Folding, sorting and degradation	22	4	13	29	17	38
Replication and repair	2	2	2	22	2	25
Environmental information processing						
Membrane transport	18	6	13	40	10	47
Signal transduction	11	1	9	8	10	13
Cellular processes						
Cell motility	12	0	15	2	18	3
Cell division	0	3	0	9	0	10
Genes total	273		739		798	

[a] Pathways are according to the KEGG database (www.genome.jp/kegg/).

[b] Number of genes involved in corresponding pathways. Only genes with fold changes of at least < 0.5 and > 2 between two conditions were used for analysis. +: induced; - repressed.

As expected, transcriptional profiling of *P. aeruginosa* cells adapting to stationary phase revealed major changes in expression of genes, which are part of the translation machinery, see tab. 3.8. Hence, genes highly repressed during transition and stationary phase encode ribosomal proteins and translation factors, see Appendix.

Transcriptome analyses also showed growth phase related changes in expression of genes involved in transcription. In accordance, genes found repressed in transition and stationary phase encoded proteins essential for transcription, *e. g.* RNA polymerase, elongation and termination factors and several sigma factors, see Appendix. Interestingly, genes found induced in adapting and resting cells mostly encoded transcriptional regulators. Consistent with high cell densities, the two quorum sensing regulators encoding genes *lasR* and *rhlR* as well as genes involved in regulation of quorum sensing, *e. g*. *vfr*, *mvaT* and *rslA*, were found induced in transition and stationary phase (Venturi, 2006). Most importantly, transcription of the global anaerobic regulator *anr* increased in transition phase indicating oxygen limitation. In this context, expression of the *arcDABC* operon encoding the genes essential for arginine fermentation was found induced during transition phase. Since transcription of these genes depends on Anr, this clearly indicated oxygen limiting conditions in transition phase (Vander Wauven *et al.*, 1984).

Expression of most of genes involved in amino acid metabolism was found decreased in transition and stationary phase indicating downregulation of overall amino acid biosynthesis. Further, a large number of genes encoding proteins acting in carbohydrate metabolism were found differentially expressed. Genes encoding proteins catalyzing the oxidation of glucose via Entner-Doudoroff pathway and glycolysis as well as genes involved in citrate cycle and pentose phosphate pathway were found downregulated during early and late stationary phase, see Appendix.

Most of genes function in energy metabolism increased upon entry into the transition phase. Interestingly, expression of the *nap* operon encoding the periplasmic nitrate reductase and additional genes previously found induced in response to low oxygen conditions in *P. aeruginosa* such as the nitrite reductase operon *nirSMC* (Alvarez-Ortega and Harwood, 2007) increased in transition phase, see Appendix. Expression of the *nap* genes was also found dependent on quorum sensing (Schuster *et al.*, 2003; Wagner *et al.*, 2003). All this is in agreement with induction of quorum sensing associated genes and oxygen limiting conditions as indicated by

induction of *anr* in transition phase as mentioned above. By comparing stationary and exponential phase, more genes were found repressed in resting cells. Consistent with low energy production during stationary phase, genes encoding proteins catalyzing ATP synthesis and NADH generation were found downregulated. Genes involved in cofactor, vitamin and nucleotide metabolism as well as genes coding for membrane transport proteins were additionally found differentially expressed by comparing different modes of growth. The majority of these genes were found downregulated during stationary phase. For example, expression of *hemL* and *hemF*, both involved in heme biosynthesis was found decreased in cells of the early and later stationary phase, see Appendix. Additionally, genes essential for vitamin synthesis, *e. g.* thiamine, riboflavine and nicotinate, were found decreased during transition and stationary phase, see Appendix.

Minor changes were detected for transcripts encoding proteins involved in glycan biosynthesis, metabolism of other amino acids and lipids, signal transduction, cell motility and cell division. Interestingly, genes associated with cell motility such as genes encoding proteins catalyzing flagellar biosynthesis as well as genes encoding aero - and chemotaxis transducers were found induced in stationary phase.

In summary, overall expression of genes encoding proteins acting in central metabolism and genetic information processing of *P. aeruginosa* was found highly induced during exponential phase. As a result of oxygen and nutrient limiting conditions during stationary phase, cellular processes like transcription, translation and replication as well as amino acid and carbohydrate metabolism were downregulated. In this context, genes coding for cell division related proteins were found repressed in transition and stationary phase. Additionally, transcription of genes involved in environmental information processing such as membrane transport and signal transduction was found induced in transition phase, but expression decreased in stationary phase implying importance during adaptation. In contrast, genes encoding proteins essential for cell motility were found upregulated in adapting and resting cells representing one survival strategy during stress phases.

3.2.1.5 Combination of metabolomics and transcriptomics

For combination of metabolome and transcriptome data on the cellular response of *P. aeruginosa* to changing growth phases, only metabolites and genes involved in *P. aeruginosa* metabolism were used. Fig. 3.11 represents an overview of pathways

mostly affected by changing growth conditions on metabolic and transcriptional level including the number of metabolites and genes found changing over time.

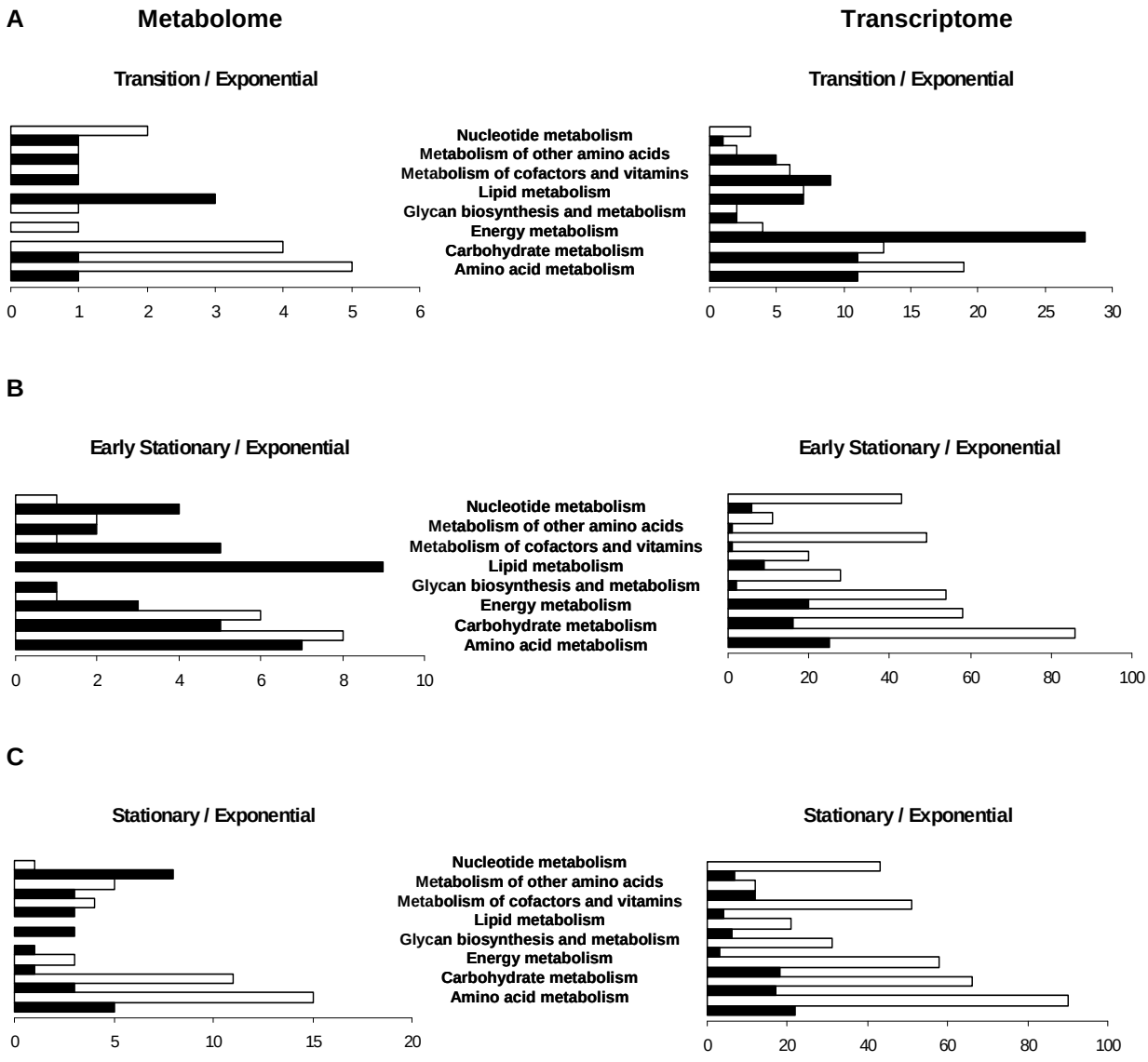


Figure 3.11: Growth phase related changes of the *P. aeruginosa* metabolism determined by metabolome and transcriptome analyses.

Transcriptome and metabolome data of *P. aeruginosa* PAO1 wild type cells of the transition phase, the early and stationary phase were compared with data from exponential grown cells. Metabolites and genes were grouped according to the KEGG database. Only metabolites and genes involved in *P. aeruginosa* metabolism were used for combination of metabolome and transcriptome data. Black: increased; white: reduced.

By comparing cells of the transition phase with cells from the exponential growth phase, metabolome analysis revealed highest differences in amino acid,

carbohydrate and lipid metabolism, see fig. 3.11 A. Whereas amino acid biosynthesis and carbohydrate production decreased within incubation time, lipid metabolism is upregulated during transition phase between exponential and stationary phase indicating degradation. On the other hand, transcriptome analysis showed highly active energy metabolism during transition phase and identified genes whose expression is induced in response to low oxygen conditions.

Comparison of early stationary and exponential phase by the use of metabolomics showed significant changes in lipid and nucleotide metabolism as well as metabolism of cofactors and vitamins, all found increased in resting cells, see fig. 3.11 B. Accumulation of these metabolites indicated degradation of lipids, nucleotides and cofactors, which might be beneficial for surviving under stress conditions like nutrient and energy limitation. For example, detection of fatty acids and its derivates indicated energy generation via degradation of membrane lipids in stationary phase. However, microarray analysis showed that expression of genes involved in synthesis of these metabolites decreased during stationary phase. Additionally, transcriptome analysis clearly showed downregulation of amino acid and carbohydrate metabolism during early stationary phase. On metabolic level, no relevant changes were observed by just comparing the number of metabolites grouped in these pathways. A more detailed view showed that this is because of a number of metabolites produced via degradation processes probably sustaining survival during stress periods, *e. g.* arginine degradation. On the other hand, in contrast to genes coding for one protein, metabolites are often connected to several pathways, which makes it difficult to detect clear differences.

Both, metabolomics and transcriptomics, showed highest differences in growth phase related changes by comparing stationary and exponential phase. Consistent with low energy production caused by oxygen and nutrient limitation, central pathways like amino acid and carbohydrate metabolism were found decreased during late stationary phase on both transcriptional and metabolic level, see fig. 3.11 C. Generally, most of the genes involved in *P. aeruginosa* metabolism were downregulated in stationary phase. Note that nucleotide components were found accumulated during stationary phase as revealed by metabolome analyses demonstrating nucleic acid degradation.

A more detailed overview representing both metabolic and transcriptional changes of the *P. aeruginosa* central carbohydrate metabolism is given in fig. 3.12.

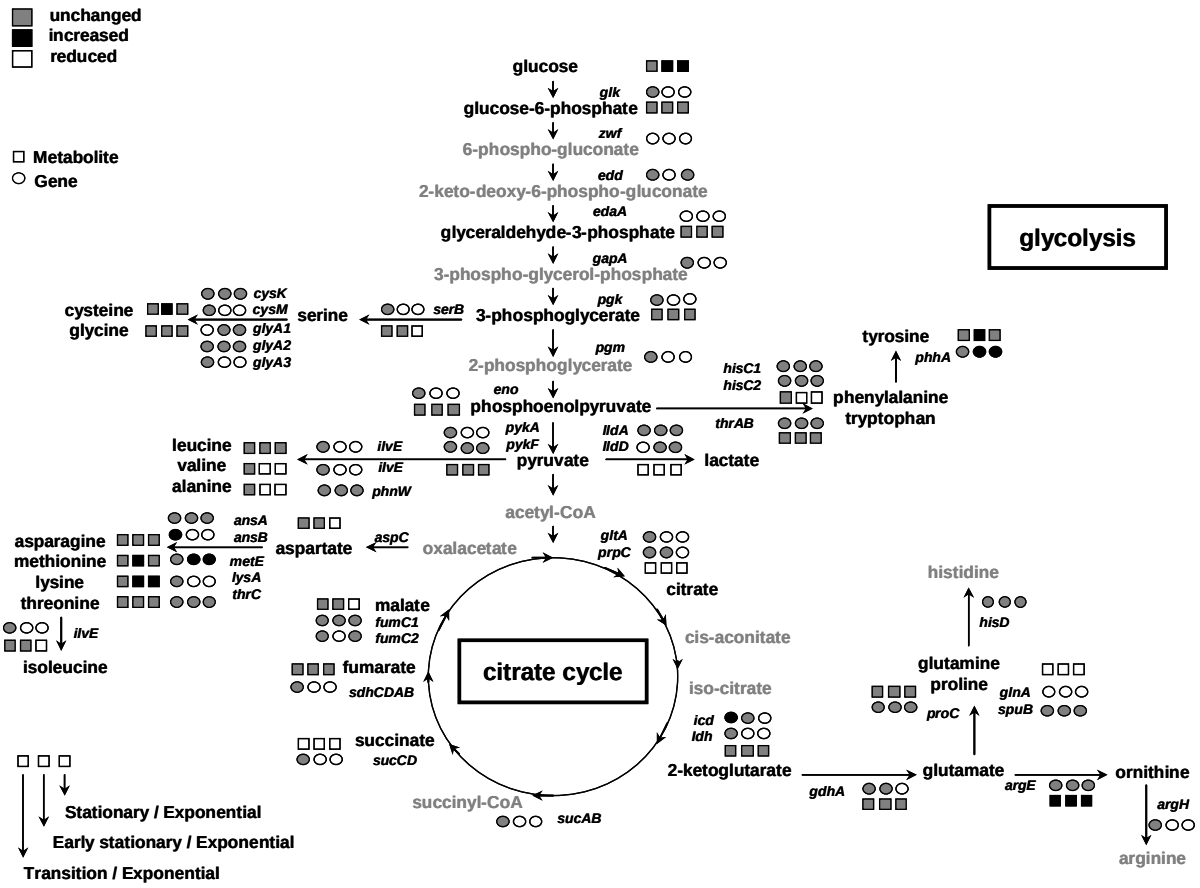


Figure 3.12: Schematic representation of growth phase associated metabolic and transcriptional changes of *P. aeruginosa* central carbohydrate metabolism.

Cells of the transition, early and late stationary phase were compared with cells of the exponential growth phase, respectively. Metabolites and genes were colored according to the corresponding growth phase in which they were mostly upregulated. Grey boxes/circles: unchanged; black boxes/circles: increased and white boxes/circles: reduced in the corresponding growth phase.

As shown in fig. 3.12, transcriptome analyses clearly showed decreased expression of genes involved in oxidation of glucose via the Entner-Doudoroff pathway and glycolysis during transition and stationary phase, but none of the corresponding intermediates was detected by metabolome analyses.

Additionally, detection of citrate and malate as well as several genes encoding enzymes of the citrate cycle demonstrated downregulation over time on both metabolic and transcriptional level. Conversely, expression of *fumC2* encoding the fumarate hydratase was found induced in early and late stationary phase.

Further, metabolome analysis showed downregulation of 5-aminolevulinate synthesis upon entry into transition phase and in stationary phase. However, transcriptome analysis showed no significant changes of *hemA* transcription, but

nevertheless revealed decreased expression of *hemL* in early and late stationary cells.

Levels of ornithine were found increased during transition and stationary phase. Since no changes of the biosynthetic gene *argE* could be observed, this clearly indicated accumulation via arginine fermentation, which is upregulated under low oxygen conditions. However, expression of the *arcDABC* genes was only found induced in transition phase implying that the enzymes of the arginine deiminase pathway are stably maintained after production during transition phase.

Lysine was found in high amounts in resting cells, although expression of the corresponding gene *lysA* decreased in stationary phase. Additionally, detection of cysteine in early stationary phase is in disagreement with expression of the subsequent gene *cysM* which was found induced in growing cells and with transcription of *cysK* not changed over time. However, no correlation between gene expression and metabolite synthesis was also observed for alanine, aspartate, glutamate, methionine and phenylalanine.

Finally, combination of transcriptomics and metabolomics provided a global picture of the *P. aeruginosa* metabolism during adaptation from exponential to stationary phase, which is important for growth and survival under natural conditions, and identified growth phase specific genes and metabolites. Metabolome and transcriptome analyses are in good agreement referring to the central carbohydrate metabolism of *P. aeruginosa* demonstrating overall downregulation of carbohydrate and amino acid metabolism over time, see fig. 3.11. Nevertheless, gene expression and metabolite concentrations did not correlate in general as it was shown in fig. 3.12. This might be due to several cellular processes (mRNA stability, translational control, protein modification and degradation). Interestingly, metabolome analyses indicated degradation of various compounds in adapting and resting cells, which might be important for survival of bacteria. Note, GC-MS and microarrays are restricted to a number of unknown metabolites and genes and thus cannot reflect the complete situation. Consequently, it seems unavoidable to integrate additional data generated by proteomics and other high-throughput metabolomics like NMR and LC-MS. Another point is that in contrast to genes and proteins, several metabolites are connected to different pathways. With the help of flux balance analysis (FBA) it will be possible to follow up metabolites and their routes in metabolism completing the picture of a cellular system.

4 SUMMARY AND OUTLOOK

4.1 SUMMARY

Central regulatory strategies of the pathogenic bacterium *Pseudomonas aeruginosa* were investigated at the gene regulatory and metabolic level.

In the first part of this thesis, three open questions concerning the major anaerobic regulatory systems of *P. aeruginosa*, Anr, Dnr and NarXL, were experimentally examined. Transcriptome analyses of the *P. aeruginosa* wild type and a *narL* mutant defective in the nitrate response regulator identified 42 genes as members of the NarL regulon. Importantly, NarL induces expression of the nitrate reductase operon *narK$_1$K$_2$GHJI* and of genes involved in the biosynthesis of enzymatic cofactors and represses transcription of the *rhl* quorum sensing genes indicating an interaction of these two regulatory networks both associated with pathogenicity. Northern blot analysis and promoter *lacZ* reporter gene studies confirmed NarL mediated repression of the arginine fermentation operon *arcDABC*. A putative NarL binding site in the promoter was functionally determined. Obviously, NarXL coordinates the anaerobic energy metabolism via induction of nitrate respiration and repression of less efficient arginine fermentation. Promoter *lacZ* reporter gene studies of the Anr-dependent *arcD* and Dnr-dependent *nirS* promoter clearly demonstrated that the downstream region of the Anr binding site consisting of the - 10 region mediates Anr and Dnr specific gene regulation and that Dnr specificity further requires the Anr binding site upstream region indicating an involvement of additional regulators.

In the second part, a reproducible method for the extraction and measurement of about 150 *P. aeruginosa* metabolites by GC-MS was successfully established in cooperation with the group of Professor Schomburg. Metabolome and transcriptome analyses of the *P. aeruginosa* wild type revealed several pathways affected by changes in growth phase and showed overall downregulation of the carbohydrate and amino acid metabolism in stationary phase at both levels. However, gene expression and metabolite concentration do not correlate in general. Interestingly, GC-MS analyses identified various new cellular responses not found with microarrays, *e. g.* production of the polyamine cadaverine related to pathogenicity. Thus, metabolomics is a potential complement to other “omics” techniques allowing systematic studies of regulatory and metabolic networks of *P. aeruginosa*.

4.2 OUTLOOK

Further experiments on the anaerobic key regulators Anr, Dnr and NarL of *P. aeruginosa* will be required to sum up results from this work.

NarL-dependence of genes identified by microarray analyses has to be verified for direct regulation by NarL.

- Promoter regions of these genes can be inspected for putative NarL binding sites and further functionally determine by using mutational analysis and promoter *lacZ* reporter gene testing.

Promoter regions up- and downstream the Anr/Dnr binding sites have to be investigated in detail in order to define specific elements essential for Anr and Dnr specificity.

- It has to be proven, whether Anr and Dnr specificity is mediated via different sigma factors by using mutants defective in corresponding genes or by mutational analysis of the - 10 region of Anr/Dnr dependent promoters.
- It has to be checked if another transcriptional regulator is involved in controlling expression of Dnr-dependent genes.

Establishment of a GC-MS based method offers new possibilities for systematical studies of *P. aeruginosa* by using the full set of “omics” techniques, which will be necessary to understand the cell as whole system and helpful for investigation of conditions associated with pathogenicity. Based on this work, it will be beneficial to perform further experiments for extension of obtained results.

- Proteome analysis on growth phase related changes should be carried out and combined with metabolome and transcriptome data.
- Unknown metabolites detected by GC-MS have to be identified for extension of the currently available metabolite spectrum of *P. aeruginosa*.

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6 APPENDIX

Gene ID	Gene name	Function	Additional Pathway/Class	Ratio EE/LE	EE/ES	EE/MS
Amino acid metabolism						
PA0036	*trpB*	tryptophan synthase beta chain		9.28	8.29	11.43
PA4695	*ilvH*	acetolactate synthase isozyme III small subunit	Metabolism of cofactors and vitamins	5.12	8.57	21.77
PA4694	*ilvC*	ketol-acid reductoisomerase	Metabolism of cofactors and vitamins	4.09	4.05	10.74
PA4696	*ilvI*	acetolactate synthase large subunit	Metabolism of cofactors and vitamins	4.01	5.78	9.81
PA0904	*lysC*	aspartate kinase alpha and beta chain		3.98	7.27	8.89
PA0654	*speD*	S-adenosylmethionine decarboxylase proenzyme		3.82	4.28	4.32
PA0035	*trpA*	tryptophan synthase alpha chain		3.43	3.12	3.06
PA5119	*glnA*	glutamine synthetase	Glycan biosynthesis and metabolism Energy metabolism Signal transduction	2.63	3.81	6.17
PA3537	*argF*	Ornithine carbamoyltransferase, anabolic		2.50	3.33	3.81
PA3118	*leuB*	3-isopropylmalate dehydrogenase		2.36	3.06	4.20
PA5415	*glyA1*	serine hydroxymethyltransferase	Metabolism of other amino acids	2.32		
PA3120	*leuD*	3-isopropylmalate dehydratase small subunit		2.28		
PA5035	*gltD*	glutamate synthase subunit beta	Energy metabolism	2.09	2.27	2.68
PA0390	*metX*	homoserine O-acetyltransferase	Energy metabolism	2.08	4.37	4.04
PA5410	*gbcA*	gbcA		2.04		
PA5419	*soxG*	sarcosine oxidase gamma subunit		2.02		
PA3121	*leuC*	sopropylmalate isomerase large subunit		2.00		
PA1010	*dapA*	dihydrodipicolinate synthase			7.38	12.52
PA4846	*aroQ1*	3-dehydroquinate dehydratase			6.95	11.77
PA3525	*argG*	argininosuccinate synthase			6.61	9.94
PA4602	*glyA3*	serine hydroxymethyltransferase	Metabolism of other amino acids		6.22	9.39
PA1750		phospho-2-dehydro-3-deoxyheptonate aldolase			5.57	10.01
PA3151	*hisF2*	imidazole glycerol phosphate synthase subunit HisF			4.96	5.34
PA5013	*ilvE*	branched-chain amino acid aminotransferase	Metabolism of cofactors and vitamins		3.96	8.65
PA3736	*hom*	homoserine dehydrogenase			3.93	3.39
PA0316	*serA*	D-3-phosphoglycerate dehydrogenase			3.90	3.71
PA4449	*hisG*	ATP phosphoribosyltransferase catalytic subunit			3.87	5.76
PA1757	*thrH*	homoserine kinase			3.82	2.47
PA4484	*gatB*	aspartyl/glutamyl-tRNA amidotransferase subunit B			3.63	4.55
PA5263	*argH*	argininosuccinate lyase			3.53	3.90
PA3152	*hisH2*	imidazole glycerol phosphate synthase subunit HisH			3.13	2.82
PA0932	*cysM*	cysteine synthase B	Metabolism of other amino acids Energy metabolism		3.36	2.60
PA4482	*gatC*	aspartyl/glutamyl-tRNA amidotransferase subunit C			3.23	2.86
PA4731	*panD*	aspartate alpha-decarboxylase	Metabolism of other amino acids		3.12	4.31
PA4007	*proA*	gamma-glutamyl phosphate reductase			3.01	3.28
PA3166	*pheA*	chorismate mutase			2.99	2.92
PA4483	*gatA*	aspartyl/glutamyl-tRNA amidotransferase subunit A			2.98	4.86

PA3139		aromatic amino acid aminotransferase			2.94	2.86
PA5140	*hisF1*	imidazole glycerol phosphate synthase subunit HisF			2.93	2.74
PA4758	*carA*	carbamoyl phosphate synthase small subunit	Nucleotide metabolism		2.88	2.79
PA5277	*lysA*	diaminopimelate decarboxylase			2.85	3.43
PA3117	*asd*	aspartate-semialdehyde dehydrogenase			2.80	2.95
PA4588	*gdhA*	glutamate dehydrogenase	Energy metabolism		2.71	2.84
PA4960	*serB*	probable phosphoserine phosphatase			2.71	2.84
PA3167	*serC*	phosphoserine aminotransferase	Metabolism of cofactors and vitamins		2.62	2.98
PA5142	*hisH1*	glutamine amidotransferase			2.58	2.86
PA0649	*trpG*	anthranilate synthase component II			2.52	2.34
PA4957	*psd*	phosphatidylserine decarboxylase	Lipid metabolism		2.50	2.82
PA0331	*ilvA1*	threonine dehydratase			2.48	2.14
PA0430	*metF*	5,10-methylenetetrahydrofolate reductase			2.43	2.49
PA5429	*aspA*	aspartate ammonia-lyase	Energy metabolism		2.37	2.25
PA5141	*hisA*	phosphoribosylformimino-5-aminoimidazole carboxamide			2.37	
PA4402	*argJ*	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein			2.25	2.00
PA5278	*dapF*	diaminopimelate epimerase			2.18	2.57
PA3107	*metZ*	O-succinylhomoserine sulfhydrylase			2.12	2.26
PA3666	*dapD*	tetrahydrodipicolinate succinylase			2.08	2.13
PA1162	*dapE*	succinyl-diaminopimelate desuccinylase			2.06	
PA5025	*metY*	O-acetylhomoserine aminocarboxypropyltransferase			2.05	2.14
PA5323	*argB*	acetylglutamate kinase			2.02	2.26
PA5067	*hisE*	phosphoribosyl-ATP pyrophosphatase				3.75
PA0651	*trpC*	indole-3-glycerol-phosphate synthase	Signal transduction			2.64
PA3792	*leuA*	2-isopropylmalate synthase	Carbohydrate metabolism			2.59
PA5203	*gshA*	glutamate--cysteine ligase	Carbohydrate metabolism			2.33
PA5080		prolyl aminopeptidase				2.27
PA5038	*aroB*	3-dehydroquinate synthase				2.16
PA5066	*hisI*	phosphoribosyl-AMP cyclohydrolase				2.07
PA0546	*metK*	S-adenosylmethionine synthetase	Metabolism of other amino acids	0.50	3.28	5.57
PA1337	*ansB*	glutaminase-asparaginase	Metabolism of other amino acids Energy metabolism	0.50	2.24	4.56
PA1642	*selD*	selenophosphate synthetase	Metabolism of other amino acids	0.50		
PA0432	*sahH*	S-adenosyl-L-homocysteine hydrolase	Metabolism of other amino acids	0.45	2.63	5.62
PA5172	*arcB*	ornithine carbamoyltransferase		0.42		4.01
PA5304	*dadA*	D-amino acid dehydrogenase small subunit	Energy metabolism	0.41	0.11	0.09
PA5171	*arcA*	arginine deiminase		0.37		2.66
PA5173	*arcC*	carbamate kinase	Nucleotide metabolism Energy metabolism	0.32		
PA3418	*ldh*	leucine dehydrogenase		0.21	0.02	0.01
PA2442	*gcvT2*	glycine cleavage system protein T2	Energy metabolism		0.35	
PA0895	*aruC*	bifunctional N-succinyldiaminopimelate-aminotransferase/acetylornithine transaminase protein			0.29	0.25
PA5098	*hutH*	histidine ammonia-lyase	Energy metabolism		0.29	
PA2015	*liuA*	putative isovaleryl-CoA dehydrogenase			0.28	0.19
PA0870	*phhC*	aromatic amino acid aminotransferase			0.26	0.26

PA5213	*gcvP1*	glycine dehydrogenase			0.25	0.43
PA3459		glutamine amidotransferase	Energy metabolism		0.25	0.26
PA1927	*metE*	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase			0.21	0.05
PA2443	*sdaA*	L-serine dehydratase			0.18	0.21
PA0871	*phhB*	pterin-4-alpha-carbinolamine dehydratase			0.18	0.19
PA2007	*maiA*	maleylacetoacetate isomerase			0.17	0.37
PA2250	*lpdV*	dihydrolipoamide dehydrogenase	Carbohydrate metabolism		0.14	0.13
PA2008	*fahA*	fumarylacetoacetase			0.12	0.37
PA2248	*bkdA2*	2-oxoisovalerate dehydrogenase (beta subunit)			0.08	0.09
PA2009	*hmgA*	homogentisate 1,2-dioxygenase			0.05	0.15
PA5100	*hutU*	urocanate hydratase			0.05	0.04
PA2247	*bkdA1*	2-oxoisovalerate dehydrogenase			0.04	0.05
PA3415		branched-chain alpha-keto acid dehydrogenase subunit E2	Carbohydrate metabolism		0.03	0.02
PA0872	*phhA*	phenylalanine 4-monooxygenase			0.02	0.01
PA0865	*hpd*	4-hydroxyphenylpyruvate dioxygenase			0.01	0.01
PA5399	*dgcB*	DgcB, Dimethylglycine catabolism				0.48
Carbohydrate metabolism						
PA2321		gluconokinase		36.26	54.17	46.13
PA5435		pyruvate carboxylase subunit B		8.74	8.09	7.81
PA2265		gluconate dehydrogenase		6.23	2.96	4.11
PA3182		6-phosphogluconolactonase		4.59	15.96	15.09
PA3183	*zwf*	glucose-6-phosphate 1-dehydrogenase		3.49	21.18	29.25
PA3181	*edaA*	2-keto-3-deoxy-6-phosphogluconate aldolase	Amino acid metabolism	3.06	8.76	11.90
PA5046		malic enzyme		2.76	5.93	5.99
PA5111	*gloA3*	lactoylglutathione lyase		2.36		
PA4748	*tpiA*	triosephosphate isomerase		2.26	13.44	18.48
PA1589	*sucD*	succinyl-CoA synthetase subunit alpha		2.11	8.53	18.05
PA1338	*ggt*	gamma-glutamyltranspeptidase precursor	Metabolism of other amino acids	2.06		2.30
PA3195	*gapA*	glyceraldehyde 3-phosphate dehydrogenase			14.31	9.33
PA5015	*aceE*	pyruvate dehydrogenase subunit E1	Amino acid metabolism		12.92	19.99
PA1582	*sdhD*	succinate dehydrogenase (D subunit)	Energy metabolism		10.88	45.24
PA1581	*sdhC*	succinate dehydrogenase (C subunit)	Energy metabolism		9.93	25.83
PA1588	*sucC*	succinyl-CoA synthetase subunit beta			8.64	27.79
PA1583	*sdhA*	succinate dehydrogenase flavoprotein subunit	Energy metabolism		5.97	19.76
PA3148	*wbpI*	UDP-N-acetylglucosamine 2-epimerase WbpI			5.74	4.95
PA5016	*aceF*	dihydrolipoamide acetyltransferase	Amino acid metabolism Energy metabolism		5.64	8.57
PA3635	*eno*	phosphopyruvate hydratase			5.31	5.71
PA1584	*sdhB*	succinate dehydrogenase (B subunit)	Energy metabolism		5.12	16.65
PA4450	*murA*	UDP-N-acetylglucosamine 1-carboxyvinyltransferase			4.90	7.81
PA5163	*rmlA*	glucose-1-phosphate thymidylyltransferase			4.86	10.12
PA1770	*ppsA*	phosphoenolpyruvate synthase			4.45	7.53
PA1585	*sucA*	alpha-ketoglutarate decarboxylase			4.44	14.81
PA4333	*fumA*	probable fumarase			4.12	7.13
PA2624	*idh*	isocitrate dehydrogenase			3.97	5.49
PA3193	*glk*	glucokinase			3.92	3.76

PA1580	*gltA*	citrate synthase			3.75	30.84
PA0548	*tktA*	transketolase			3.49	4.35
PA1586	*sucB*	dihydrolipoamide acetyltransferase			3.21	6.44
PA3818	*suhB*	extragenic suppressor protein SuhB			3.21	3.46
PA5192	*pckA*	phosphoenolpyruvate carboxykinase			3.12	4.85
PA0552	*pgk*	phosphoglycerate kinase			3.08	3.28
PA4329	*pykA*	pyruvate kinase	Nucleotide metabolism		3.01	3.05
PA1796	*folD*	5,10-methylene-tetrahydrofolate dehydrogenase / cyclohydrolase			2.94	2.40
PA1587	*lpdG*	dihydrolipoamide dehydrogenase	Amino acid metabolism		2.81	4.94
PA3001		glyceraldehyde-3-phosphate dehydrogenase			2.75	8.63
PA1787	*acnB*	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase			2.73	13.35
PA2023	*galU*	UTP--glucose-1-phosphate uridylyltransferase			2.72	5.28
PA5322	*algC*	phosphomannomutase AlgC			2.54	2.69
PA4640	*mqoB*	malate:quinone oxidoreductase			2.44	5.68
PA5110	*fbp*	fructose-1,6-bisphosphatase			2.41	3.61
PA0330	*rpiA*	ribose-5-phosphate isomerase A			2.38	3.17
PA3172		phosphoglycolate phosphatase			2.27	2.52
PA5162	*rmlD*	dTDP-4-dehydrorhamnose reductase			2.24	
PA3194	*edd*	phosphogluconate dehydratase			2.21	
PA5131	*pgm*	phosphoglyceromutase			2.19	2.81
PA5453	*gmd*	GDP-mannose 4,6-dehydratase			2.19	2.02
PA4749	*glmM*	phosphoglucosamine mutase			2.09	2.57
PA5552	*glmU*	glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate uridyltransferase			2.08	2.74
PA4314	*purU1*	formyltetrahydrofolate deformylase			2.04	
PA5161	*rmlB*	dTDP-D-glucose 4,6-dehydratase			2.02	2.45
PA3471		malate dehydrogenase			2.01	2.32
PA0795	*prpC*	methylcitrate synthase				6.18
PA3524	*gloA1*	lactoylglutathione lyase				2.80
PA0835	*pta*	phosphate acetyltransferase				2.64
PA5164	*rmlC*	dTDP-4-dehydrorhamnose 3,5-epimerase				2.21
PA0954		acylphosphatase		0.48		
PA0482	*glcB*	malate synthase G		0.46		2.95
PA4876	*osmE*	DNA-binding transcriptional activator OsmE		0.39	0.03	0.04
PA2025	*gor*	glutathione reductase	Amino acid metabolism	0.38	0.20	0.25
PA4771	*lldD*	L-lactate dehydrogenase		0.35		
PA1562	*acnA*	aconitate hydratase		0.30	0.10	0.13
PA2623	*icd*	isocitrate dehydrogenase		0.26		3.91
PA5427	*adhA*	alcohol dehydrogenase	Energy metabolism	0.22	3.63	3.71
PA2119		alcohol dehydrogenase (Zn-dependent)	Lipid metabolism Amino acid metabolism	0.21		
PA4236	*katA*	catalase		0.14		
PA3479	*rhlA*	rhamnosyltransferase chain A		0.11		
PA2290	*gcd*	glucose dehydrogenase			0.43	
PA4733	*acsB*	acetyl-CoA synthetase			0.40	
PA1950	*rbsK*	ribokinase			0.34	0.42
PA3568	*ymmS*	probable acetyl-coa synthetase			0.34	

PA1997		acetoacetyl-CoA synthetase			0.23	0.21
PA0854	*fumC2*	fumarate hydratase			0.22	0.40
PA0887	*acsA*	acetyl-CoA synthetase			0.19	4.17
PA2634	*aceA*	isocitrate lyase			0.19	2.01
PA2249	*bkdB*	branched-chain alpha-keto acid dehydrogenase (lipoamide component)	Amino acid metabolism		0.14	0.14
PA0838		glutathione peroxidase			0.13	0.20
PA2003	*bdhA*	3-hydroxybutyrate dehydrogenase				0.49
PA2153	*glgB*	glycogen branching enzyme				0.42
PA0447	*gcdH*	glutaryl-CoA dehydrogenase	Lipid metabolism			0.25
Transcription						
PA2259	*ptxS*	transcriptional regulator PtxS		4.73	2.94	3.23
PA2320	*gntR*	transcriptional regulator GntR		3.58	4.74	4.94
PA4269	*rpoC*	DNA-directed RNA polymerase beta chain	Nucleotide metabolism	2.87	6.75	11.98
PA4270	*rpoB*	DNA-directed RNA polymerase beta chain		2.63	4.59	5.45
PA4238	*rpoA*	DNA-directed RNA polymerase alpha chain	Nucleotide metabolism	2.56	9.47	26.65
PA5274	*rnk*	nucleoside diphosphate kinase regulator		2.02	3.45	4.81
PA4665	*prfA*	peptide chain release factor 1		2.01	3.12	2.87
PA4275	*nusG*	transcription antitermination protein NusG			7.97	12.55
PA0576	*rpoD*	transkription sigma factor Rpo			5.07	17.84
PA4745	*nusA*	transcription elongation factor NusA			5.05	4.88
PA4723	*dksA*	suppressor protein DksA			3.72	8.13
PA5239	*rho*	transcription termination factor Rho			3.61	4.81
PA1776	*sigX*	ECF sigma factor SigX			2.91	5.79
PA4057	*nrdR*	NrdR			2.78	3.36
PA5562	*spoOJ*	chromosome partitioning protein Spo0J			2.56	3.44
PA4755	*greA*	transcription elongation factor GreA			2.24	
PA0527	*dnr*	transcriptional regulator Dnr			2.16	2.12
PA0612	*ptrB*	Repressor PtrB			2.09	2.08
PA4944	*hfq*	Hfq				5.66
PA5337	*rpoZ*	DNA-directed Transkription subunit omega				2.89
PA4462	*rpoN*	transkription factor sigma-54				2.57
PA3583	*glpR*	glycerol-3-phosphate regulon repressor				2.41
PA4778		transcriptional regulator				2.20
PA4764	*fur*	ferric uptake regulation protein				2.12
PA1097	*fleQ*	transcriptional regulator FleQ		0.50		
PA1754	*cysB*	transcriptional regulator CysB		0.50		
PA1003	*mvfR*	transcriptional regulator MvfR		0.48	0.38	0.47
PA1544	*anr*	transcriptional regulator Anr		0.44		4.22
PA0652	*vfr*	cAMP-regulatory protein		0.43	2.90	6.81
PA0376	*rpoH*	transkription factor sigma-32		0.37		
PA2591		transcriptional regulator		0.34		
PA1431	*rsaL*	regulatory protein RsaL		0.31	0.30	
PA3477	*rhlR*	transcriptional regulator RhlR		0.26	0.48	
PA3385	*amrZ*	alginate and motility regulator Z		0.20	0.10	0.09
PA2622	*cspD*	cold-shock protein CspD		0.14	0.18	0.22
PA0942		transcriptional regulator			0.49	0.36
PA4315	*mvaT*	transcriptional regulator MvaT			0.47	0.47

PA4147	*acoR*	transcriptional regulator AcoR			0.43	0.18
PA5499	*np20*	transcriptional regulator np20			0.35	
PA0610	*prtN*	transcriptional regulator PrtN			0.29	0.24
PA2896		transkription sigma factor			0.28	0.27
PA2016	*liuR*	regulator of liu genes			0.26	0.14
PA2246	*bkdR*	transcriptional regulator BkdR			0.24	0.33
PA1430	*lasR*	transcriptional regulator LasR			0.24	0.24
PA0815		transcriptional regulator			0.23	0.21
PA5105	*hutC*	histidine utilization repressor HutC			0.18	0.25
PA5356	*glcC*	DNA-binding transcriptional regulator GlcC			0.15	0.16
PA0762	*algU*	Transkription sigma factor AlgU			0.08	0.07
PA1826		transcriptional regulator				0.50
PA3587	*metR*	transcriptional regulator MetR				0.49
PA0289	*gpuR*	transcriptional activator GpuR				0.45
Translation						
PA4255	*rpmC*	50S ribosomal protein L29		4.43	12.55	14.15
PA4272	*rplJ*	50S ribosomal protein L10		4.19	14.65	83.82
PA4273	*rplA*	50S ribosomal protein L1		3.62	11.81	54.03
PA4244	*rplO*	50S ribosomal protein L15		3.60	10.85	25.14
PA4254	*rpsQ*	30S ribosomal protein S17		3.48	12.25	88.42
PA4261	*rplW*	50S ribosomal protein L23		3.43	18.06	65.52
Pae_tRNA_Ala				3.31	5.23	5.80
PA4253	*rplN*	50S ribosomal protein L14		3.12	10.33	37.58
PA4258	*rplV*	50S ribosomal protein L22		3.06	11.49	33.87
PA4260	*rplB*	50S ribosomal protein L2		3.04	9.33	25.66
PA4241	*rpsM*	30S ribosomal protein S13		3.02	13.53	67.31
PA4246	*rpsE*	30S ribosomal protein S5		2.83	10.76	55.33
PA4271	*rplL*	50S ribosomal protein L7 / L12		2.83	8.44	33.84
PA4248	*rplF*	50S ribosomal protein L6		2.76	14.48	59.09
PA4262	*rplD*	50S ribosomal protein L4		2.76	15.24	54.19
PA4256	*rplP*	50S ribosomal protein L16		2.76	9.72	29.18
PA4239	*rpsD*	30S ribosomal protein S4		2.74	13.23	48.98
PA4252	*rplX*	50S ribosomal protein L24		2.73	9.26	30.08
PA4263	*rplC*	50S ribosomal protein L3		2.66	14.95	41.54
PA4240	*rpsK*	30S ribosomal protein S11		2.63	12.47	39.20
PA4249	*rpsH*	30S ribosomal protein S8		2.63	10.43	22.03
PA4247	*rplR*	50S ribosomal protein L18		2.61	11.55	48.76
PA4257	*rpsC*	30S ribosomal protein S3		2.54	9.90	53.49
PA4245	*rpmD*	50S ribosomal protein L30		2.52	10.79	66.22
PA4274	*rplK*	50S ribosomal protein L11		2.47	10.50	62.37
PA4743	*rbfA*	Translation-binding factor A		2.36	5.22	6.67
PA4259	*rpsS*	30S ribosomal protein S19		2.33	8.35	43.81
PA4935	*rpsF*	30S ribosomal protein S6		2.25	8.89	31.98
Pae_tRNA_Ile				2.23	8.38	18.93
PA3700	*lysS*	lysyl-tRNA synthetase	Amino acid metabolism	2.23	6.24	7.73
PA4932	*rplI*	50S ribosomal protein L9		2.18	5.05	7.10
PA4672	*pth*	peptidyl-tRNA hydrolase		2.13	5.74	4.95
PA4242	*rpmJ*	50S ribosomal protein L36		2.13	8.91	104.15

Pae_tRNA_Trp				2.12	9.71	7.91
PA3743	*trmD*	RNA (guanine-N(1)-)-methyltransferase		2.11	9.71	15.62
PA4266	*fusA1*	elongation factor G		2.07	11.52	47.60
PaetRNAGln				2.08	2.13	
PA4934	*rpsR*	30S ribosomal protein S18		2.07	7.45	22.85
PA4237	*rplQ*	50S ribosomal protein L17		2.07	5.63	10.40
PA2619	*infA*	translation initiation factor IF-1		2.04	6.70	8.02
PA3834	*valS*	valyl-tRNA synthetase		2.03	6.47	7.29
PA5570	*rpmH*	50S ribosomal protein L34			16.98	56.00
PA5316	*rpmB*	50S ribosomal protein L28			15.44	135.32
PA0579	*rpsU*	30S ribosomal protein S21			14.87	50.50
PA4563	*rpsT*	30S ribosomal protein S20			13.01	88.23
PA4568	*rplU*	50S ribosomal protein L21			12.27	52.36
PA3745	*rpsP*	30S ribosomal protein S16			11.41	27.47
PA4433	*rplM*	50S ribosomal protein L13			10.26	44.32
PA4264	*rpsJ*	30S ribosomal protein S10			8.81	37.60
PA4267	*rpsG*	30S ribosomal protein S7			8.65	25.02
PA3656	*rpsB*	30S ribosomal protein S2			8.65	13.29
PA4765	*omlA*	outer membrane lipoprotein OmlA precursor			7.87	18.77
PA2851	*efp*	elongation factor P			7.75	16.33
PA3655	*tsf*	elongation factor Ts			7.71	13.09
PA3744	*rimM*	16S rRNA processing protein			7.55	13.40
PA4268	*rpsL*	30S ribosomal protein S12			7.42	26.13
PA4432	*rpsI*	30S ribosomal protein S9			6.77	22.66
PA4251	*rplE*	50S ribosomal protein L5			6.69	30.29
PA4250	*rpsN*	30S ribosomal protein S14			6.25	20.91
PA4567	*rpmA*	50S ribosomal protein L27			6.05	16.67
PA4265	*tufAs*	elongation factor Tu			6.02	19.56
PA3162	*rpsA*	30S ribosomal protein S1			5.88	19.78
PA2970	*rpmF*	50S ribosomal protein L32			5.74	17.34
PA0963	*aspS*	aspartyl-tRNA synthetase	Amino acid metabolism		5.62	9.19
PA2740	*pheS*	phenylalanyl-tRNA synthetase subunit alpha	Amino acid metabolism		5.07	3.77
PA2744	*thrS*	threonyl-tRNA synthetase	Amino acid metabolism		5.05	5.54
PA4741	*rpsO*	30S ribosomal protein S15			4.75	16.99
PA0969	*tolQ*	TolQ protein			4.68	6.62
PA5049	*rpmE*	50S ribosomal protein L31			4.51	6.32
PA4744	*infB*	translation initiation factor IF-2			4.21	4.00
PA3742	*rplS*	50S ribosomal protein L19			4.09	5.37
PA1795	*cysS*	cysteinyl-tRNA synthetase	Amino acid metabolism		3.98	6.31
PA3482	*metG*	methionyl-tRNA synthetase	Amino acid metabolism Metabolism of other amino acids		3.28	3.52
PA5315	*rpmG*	50S ribosomal protein L33			3.77	9.92
PA3802	*hisS*	histidyl-tRNA synthetase	Amino acid metabolism		3.66	5.00
PA0956	*proS*	prolyl-tRNA synthetase	Amino acid metabolism		3.70	3.39
PA3653	*frr*	translation recycling factor			3.42	4.33
PA1794	*glnS*	glutaminyl-tRNA synthetase	Amino acid metabolism		3.25	4.50
PA4727	*pcnB*	poly(A) polymerase			3.10	2.75
Pae_tRNA_Gly					3.01	5.66
PA2976	*rne*	ribonuclease E			2.87	5.61

PA5051	*argS*	arginyl-tRNA synthetase	Amino acid metabolism		2.85	2.49
PA5134	*ctpA*	probable carboxyl-terminal protease			2.84	4.14
PA3987	*leuS*	leucyl-tRNA synthetase	Amino acid metabolism		2.84	3.27
PA4560	*ileS*	isoleucyl-tRNA synthetase	Amino acid metabolism		2.69	2.47
PA4850	*prmA*	ribosomal protein L11 methyltransferase			2.61	3.15
PA4742	*truB*	tRNA pseudouridine 55 synthase			2.50	2.19
PA2626	*trmU*	tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase			2.48	2.83
PA0903	*alaS*	alanyl-tRNA synthetase	Amino acid metabolism		2.45	2.46
PA4544	*rluD*	pseudouridine synthase			2.37	2.40
PA2739	*pheT*	phenylalanyl-tRNA synthetase subunit beta	Amino acid metabolism		2.37	2.26
PA3134	*gltX*	glutamyl-tRNA synthetase	Amino acid metabolism Metabolism of cofactors and vitamins		2.30	
PA0009	*glyQ*	glycyl-tRNA synthetase subunit alpha	Amino acid metabolism		2.29	2.27
PA0592	*ksgA*	rRNA (adenine-N6,N6)-dimethyltransferase			2.28	
PA2743	*infC*	translation initiation factor IF-3			2.27	4.78
PA2742	*rpmI*	50S ribosomal protein L35			2.17	5.12
PA2741	*rplT*	50S ribosomal protein L20			2.11	4.86
PA2612	*serS*	seryl-tRNA synthetase	Amino acid metabolism		2.11	3.89
Pae_tRNA_Lys					2.07	3.20
PA3657	*map*	methionine aminopeptidase			2.05	2.78
PA2755	*eco*	ecotin precursor				3.25
PA0421	*rpmE2*	50S ribosomal protein L31 type B				2.14
Pae_ tRNA_Cys				0.43	0.44	0.47
Pae_tRNA_Thr				0.35	0.29	0.33
PA3049	*rmf*	translation modulation factor		0.02	0.03	0.04
PA1122		probable peptide deformylase			0.47	
Pae_tRNA_Ser					0.40	0.40
PA3865		amino acid binding protein			0.33	0.23
Pae_tRNA_Arg					0.31	0.23
PA2071	*fusA2*	elongation factor G			0.18	0.30
PA0355	*pfpI*	protease PfpI			0.07	0.08
PA3600	*rpl36*	conserved hypothetical protein			0.03	0.48
PA1928	*rimJ*	ribosomal protein alanine acetyltransferase				0.43
Membrane transport						
PA2322		gluconate permease		58.00	52.47	50.39
PA4501	*opdD*	glycine-glutamate dipeptide porin OpdP		3.88		3.77
PA4243	*secY*	secretion protein SecY		3.71	10.82	24.95
PA5450	*wzt*	ABC subunit of A-band LPS efflux transporter		2.28	2.28	2.28
PA4898	*opdK*	vanillate porin OpdK		2.24		
PA4757	*yeaS*	conserved hypothetical		2.16	3.42	4.01
PA2760	*oprQ*	probable outer membrane protein precursor		2.05	4.78	14.87
PA3190	*gltB*	probable binding protein component of ABC sugar transporter			31.73	21.98
PA5128	*secB*	preprotein translocase subunit SecB			18.00	22.39
PA4276	*secE*	secretion protein SecE			14.65	32.19
PA3186	*oprB*	glucose/carbohydrate outer membrane porin OprB precursor			7.33	10.03

PA3822	*yaiC*	preprotein translocase subunit YajC		5.98	8.51
PA4403	*secA*	preprotein translocase subunit SecA		4.84	9.14
PA3648		outer membrane protein precursor		4.35	4.73
PA0281	*cysW*	sulfate transport protein CysW		4.38	4.85
PA4997	*msbA*	transport protein MsbA		4.33	5.71
PA0374	*ftsE*	cell division protein FtsE		4.24	4.42
PA4747	*secG*	preprotein translocase subunit SecG		4.00	7.98
PA1493	*cysP*	sulfate-binding protein of ABC transporter		3.76	8.10
PA0973	*oprL*	peptidoglycan associated lipoprotein OprL precursor		3.72	6.64
PA0768	*lepB*	signal peptidase I		3.65	6.21
PA0972	*tolB*	translocation protein TolB		3.31	5.54
PA4974	*opmH*	probable outer membrane protein precursor		3.21	4.03
PA3821	*secD*	preprotein translocase subunit SecD		3.14	3.39
PA0283	*sbp*	sulfate-binding protein precursor		3.12	3.02
PA5070	*tatC*	transport protein TatC		2.99	2.70
PA0958	*oprD*	basic amino acid, basic peptide and imipenem outer membrane porin OprD precursor		2.98	12.51
PA0373	*ftsY*	signal recognition particle receptor FtsY		2.93	3.74
PA5069	*tatB*	sec-independent translocase		2.88	3.51
PA4464	*ptsN*	nitrogen regulatory IIA protein		2.75	4.09
PA4559	*lspA*	lipoprotein signal peptidase		2.38	
PA0970	*tolR*	TolR protein		2.28	2.78
PA0302	*potG*	polyamine transport protein PotG		2.24	2.45
PA1775	*cmpX*	conserved cytoplasmic membrane protein, CmpX protein		2.21	
PA1475	*ccmA*	heme exporter protein CcmA		2.17	2.11
PA2812	*yadG*	probable ATP-binding component of ABC transporter		2.16	2.21
PA3746	*ffh*	signal recognition particle protein Ffh		2.14	
PA0303	*potH*	polyamine transport protein PotH		2.13	2.93
PA0375	*ftsX*	cell division protein FtsX		2.12	2.10
PA3820	*secF*	preprotein translocase subunit SecF		2.06	2.19
PA5479	*gltP*	glutamate/aspartate:proton symporter		2.04	2.11
PA0300	*spuD*	polyamine transport protein			3.17
PA1777	*oprF*	major porin and structural outer membrane porin OprF precursor			2.81
PA4506	*dppF*	dipeptide transporter ATP-binding subunit			2.51
PA0301	*spuE*	polyamine transport protein			2.43
PA3887	*nhaP*	Na+/H+ antiporter NhaP			2.40
PA4370	*icmP*	Insulin-cleaving metalloproteinase outer membrane protein precursor			2.32
PA0425	*mexA*	RND multidrug efflux membrane fusion protein MexA precursor			2.32
PA0337	*ptsP*	phosphoenolpyruvate-protein phosphotransferase PtsP			2.22
PA3692		outer membrane protein precursor	0.48	0.01	0.02
PA3234	*actP*	acetate permease	0.47	0.06	
PA1183	*dctA*	C4-dicarboxylate transporter DctA	0.46		
PA5068	*tatA*	twin argininte translocase protein A	0.45		
PA0888	*aotJ*	arginine/ornithine binding protein AotJ	0.43		2.13

PA3336		major facilitator transporter		0.41		
PA1861	*modC*	molybdenum transport protein ModC		0.36		
PA3526	*motY*	outer membrane protein precursor		0.34	0.17	0.29
PA4687	*hitA*	ferric iron-binding periplasmic protein HitA		0.33	0.24	
PA5170	*arcD*	arginine/ornithine antiporter		0.29	0.35	
PA1862	*modB*	molybdenum transport protein ModB		0.29		
PA0291	*oprE*	anaerobically-induced outer membrane porin OprE precursor		0.27		5.74
PA4359	*feoA*	ferrous iron transport protein A		0.27		
PA4770	*lldP*	L-lactate permease		0.21		
PA4614	*mscL*	large-conductance mechanosensitive channel		0.19	0.14	0.17
PA3790	*oprC*	putative copper transport outer membrane porin OprC precursor		0.17	0.36	
PA4067	*oprG*	outer membrane protein OprG precursor		0.13	3.72	6.96
PA1863	*modA*	molybdate-binding periplasmic protein precursor ModA		0.11	0.48	
PA1946	*rbsB*	binding protein component precursor of ABC ribose transporter			0.46	
PA3316		ABC transporter permease			0.42	0.42
PA5107	*blc*	outer membrane lipoprotein Blc			0.40	
PA0215	*madL*	malonate transporter MadL			0.39	0.50
PA5094		ABC transporter ATP-binding protein			0.32	0.47
PA0452	*slp*	probable stomatin-like protein			0.17	0.08
PA1786	*nasF/S*	conserved hypothetical protein				0.49
PA3375		ABC transporter ATP-binding protein				0.49
PA2830	*htpX*	heat shock protein HtpX				0.42
Lipid metabolism						
PA1610	*fabA*	beta-hydroxydecanoyl-ACP dehydrase		3.85	4.96	4.11
PA2862	*lipA*	lactonizing lipase precursor		2.93	0.29	
PA5351	*rubA1*	Rubredoxin 1		3.35	10.30	22.53
PA0074	*ppkA*	serine/threonine protein kinase PpkA		2.50		
PA4848	*accC*	acetyl-CoA carboxylase biotin carboxylase subunit	Carbohydrate metabolism	2.19	5.16	7.66
PA4888	*desD*	acyl-CoA delta-9-desaturase, DesB		2.04		
PA1609	*fabB*	oxoacyl-(acyl carrier protein) synthase I		2.03	2.21	3.29
PA4847	*accB*	acetyl-CoA carboxylase biotin carboxyl carrier protein subunit	Carbohydrate metabolism		6.18	10.09
PA3645	*fabZ*	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase			4.62	5.75
PA4669	*ipk*	isopentenyl monophosphate kinase			4.11	5.45
PA3803	*ispG*	gcpE; 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase			3.88	6.91
PA2967	*fabG*	3-oxoacyl-[acyl-carrier-protein] reductase			3.54	3.91
PA3299	*fadD1*	acyl-CoA synthetase			3.11	4.72
PA2584	*pgsA*	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase			3.09	3.06
PA2968	*fabD*	malonyl-CoA-[acyl-carrier-protein] transacylase			2.84	3.18
PA3639	*accA*	acetyl-CoA carboxylase carboxyltransferase subunit alpha	Carbohydrate metabolism		2.66	3.01

PA1806	*fabI*	NADH-dependent enoyl-ACP reductase			2.36	2.50
PA4557	*lytB*	4-hydroxy-3-methylbut-2-enyl diphosphate reductase			2.25	2.38
PA0341	*lgt*	prolipoprotein diacylglyceryl transferase			2.19	2.21
PA1748		enoyl-CoA hydratase	Amino acid metabolism Carbohydrate metabolism Metabolism of other amino acids		2.13	
PA4044	*dxs*	1-deoxy-D-xylulose-5-phosphate synthase			2.04	
PA0286	*desA*	delta-9 fatty acid desaturase			2.02	
PA4043	*ispA*	geranyltranstransferase				2.61
PA3286		3-oxoacyl-(acyl carrier protein) synthase III				2.11
PA4050	*pgpA*	phosphatidylglycerophosphatase A				2.03
PA3857	*pcs*	phosphatidylcholine synthase		0.46	0.44	
PA2815		acyl-CoA dehydrogenase		0.41	0.22	0.30
PA4389	*fabG*	3-ketoacyl-(acyl-carrier-protein) reductase		0.35		2.29
PA1869		Probable acyl carrier protein		0.21		
PA3334		Probable acyl carrier protein		0.19	0.37	
PA3333	*fabH2*	3-oxoacyl-(acyl carrier protein) synthase III		0.14	0.38	
PA2841		enoyl-CoA hydratase	Amino acid metabolism Carbohydrate metabolism Metabolism of other amino acids		0.47	0.47
PA2552	*acdB*	probable acyl-CoA dehydrogenase			0.47	0.33
PA1470		short chain dehydrogenase			0.40	0.37
PA4786	*fabG*	3-ketoacyl-(acyl-carrier-protein) reductase			0.38	0.43
Metabolism of cofactors and vitamins						
PA4973	*thiC*	thiamin biosynthesis protein ThiC		2.48	3.20	3.18
PA4664	*hemK*	probable methyl transferase		2.38	2.82	2.43
PA4919	*pncB1*	nicotinate phosphoribosyltransferase		2.11	2.50	2.19
PA0363	*coaD*	phosphopantetheine adenylyltransferase			6.91	11.33
PA4569	*ispB*	octaprenyl-diphosphate synthase			5.08	5.89
PA1674	*folE2*	GTP cyclohydrolase I			4.84	6.35
PA3977	*hemL*	glutamate-1-semialdehyde 2,1-aminomutase			3.99	3.38
PA3976	*thiE*	thiamine-phosphate pyrophosphorylase			3.50	6.23
PA4730	*panC*	pantoate--beta-alanine ligase	Metabolism of other amino acids		3.50	2.62
PA0381	*thiG*	thiazole synthase			3.48	3.88
PA3814	*iscS*	L-cysteine desulfurase (pyridoxal phosphate-dependent)			3.24	
PA4053	*ribE*	riboflavin synthase subunit beta			2.99	5.04
PA5118	*hiI*	thiamine biosynthesis protein ThiI			2.98	2.42
PA3812	*iscA*	Probable iron-binding protein IscA			2.94	
PA5063	*ubiE*	ubiquinone/menaquinone biosynthesis methyltransferase			2.91	3.28
PA0551	*epd*	D-erythrose 4-phosphate dehydrogenase			2.87	3.72
PA5320	*coaC*	Phosphopantothenoylcysteine synthase/(R)-4'-phospho-N-pantothenoylcysteine decarboxylase			2.85	3.65
PA4561	*ribF*	riboflavin kinase/FAD synthase			2.77	2.64
PA4280	*birA*	biotin--protein ligase			2.74	2.39
PA4006	*nadA*	nicotinic acid mononucleotide adenylyltransferase			2.65	2.60
PA3975	*thiD*	phosphomethylpyrimidine kinase			2.64	2.81

PA5065	*ubiB*	ubiquinone biosynthetic protein UbiB			2.59	2.74
PA4729	*panB*	3-methyl-2-oxobutanoate hydroxymethyltransferase			2.52	
PA3997	*lipB*	lipoyltransferase			2.39	2.87
PA0350	*folA*	dihydrofolate reductase			2.37	2.41
PA0024	*hemF*	coproporphyrinogen III oxidase			2.29	2.07
PA5237	*yigC*	conserved hypothetical protein			2.26	2.16
PA0593	*pdxA*	pyridoxal phosphate biosynthetic protein PdxA			2.12	2.07
PA2945	*cobW*	conserved hypothetical protein			2.09	2.49
PA0420	*bioA*	adenosylmethionine-8-amino-7-oxononanoate aminotransferase			2.01	2.32
PA3439	*folX*	D-erythro-7,8-dihydroneopterin triphosphate 2'-epimerase				2.43
PA3029	*moaB2*	molybdopterin biosynthetic protein B2				2.39
PA2991	*sth*	soluble pyridine nucleotide transhydrogenase				2.13
PA4054	*ribB*	bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II protein				2.00
PA1049	*pdxH*	pyridoxamine 5'-phosphate oxidase		0.41		
PA1549		cation-transporting P-type ATPase		0.40		
PA3397	*fpr*	ferredoxin--NADP+ reductase	Energy metabolism	0.39	3.69	3.98
PA5243	*hemB*	delta-aminolevulinic acid dehydratase		0.35		
PA3171	*ubiG*	3-demethylubiquinone-9 3-methyltransferase		0.34		2.34
PA1985	*pqqA*	coenzyme PQQ synthesis protein PqqA		0.11	0.02	0.29
PA1546	*hemN*	coproporphyrinogen III oxidase		0.07		
PA2103	*moeB*	molybdopterin biosynthesis protein MoeB				0.47
PA5259	*hemD*	uroporphyrinogen-III synthase				0.45
PA0501	*bioF*	8-amino-7-oxononanoate synthase				0.45
Folding, sorting and degradation						
PA5129	*grx*	glutaredoxin		2.44	10.84	17.90
PA4572	*fklB*	peptidyl-prolyl cis-trans isomerase FklB		2.38		
PA5193	*yrfI*	heat shock protein HSP33		2.24	2.78	2.36
PA1596	*htpG*	heat shock protein 90		2.04	5.49	8.71
PA1800	*tig*	trigger factor			11.87	23.30
PA4385	*groEL*	GroEL protein			6.32	15.48
PA4386	*groES*	co-chaperonin GroES			6.09	26.88
PA0594	*surA*	peptidyl-prolyl cis-trans isomerase SurA			4.76	8.36
PA1805	*ppiD*	peptidyl-prolyl cis-trans isomerase D			4.49	7.29
PA4762	*grpE*	heat shock protein GrpE			4.47	8.64
PA5054	*hslU*	heat shock protein HslU			4.38	7.13
PA1008	*bcp*	bacterioferritin comigratory protein			4.13	6.90
PA3737	*dsbC*	thiol:disulfide interchange protein DsbC			4.03	6.27
PA4176	*ppiC2*	peptidyl-prolyl cis-trans isomerase C2			3.77	6.19
PA4942	*hflK*	protease subunit HflK			2.96	3.07
PA1793	*ppiB*	peptidyl-prolyl cis-trans isomerase B			2.90	4.79
PA5240	*trxA*	thioredoxin			2.85	5.91
PA2614	*lolA*	outer-membrane lipoprotein carrier protein			2.64	3.18
PA4428	*sspA*	stringent starvation protein A			2.50	2.85
PA1801	*clpP*	ATP-dependent Clp protease proteolytic subunit			2.43	4.59
PA4768	*smpB*	SsrA-binding protein			2.37	2.08

PA3811	*hscB*	heat shock protein HscB			2.35	
PA5053	*hslV*	ATP-dependent protease peptidase subunit			2.19	2.50
PA4761	*dnaK*	molecular chaperone DnaK			2.18	3.04
PA0837	*slyD*	peptidyl-prolyl cis-trans isomerase SlyD			2.09	2.89
PA4941	*hflC*	protease subunit HflC				2.25
PA4525	*pilA*	type 4 fimbrial precursor PilA	Signal transduction			2.23
PA4061	*ybbN*	probable thioredoxin				2.15
PA0395	*pilT*	twitching motility protein PilT				2.02
PA1718	*pscE*	type III export protein PscE				2.01
PA0410	*pilI*	twitching motility protein PilI		0.49	3.04	4.67
PA0139	*ahpC*	alkyl hydroperoxide reductase subunit C		0.48		5.15
PA2618		arginyl-tRNA-protein transferase		0.47	0.03	0.03
PA2960	*pilZ*	type 4 fimbrial biogenesis protein PilZ		0.46		2.24
PA3126	*ibpA*	heat-shock protein IbpA		0.46	0.37	
PA5041	*pilP*	type 4 fimbrial biogenesis protein PilP		0.45		2.11
PA0411	*pilJ*	twitching motility protein PilJ	Signal transduction	0.44	3.25	4.70
PA5044	*pilM*	type 4 fimbrial biogenesis protein PilM		0.44		
PA0409	*pilH*	twitching motility protein PilH	Signal transduction	0.43	2.97	8.54
PA1135		chaperone protein HchA		0.43	0.16	0.31
PA2827		methionine sulfoxide reductase B		0.43	0.13	0.14
PA5040	*pilQ*	type 4 fimbrial biogenesis outer membrane protein PilQ precursor		0.42		2.24
PA2621	*clpS*	ATP-dependent Clp protease adaptor protein ClpS		0.42	0.08	0.09
PA2620	*clpA*	ATP-binding protease component ClpA		0.41	0.06	0.04
PA0408	*pilG*	twitching motility protein PilG	Signal transduction	0.41	2.83	9.95
PA0396	*pilU*	twitching motility protein PilU		0.38	0.42	
PA3221	*csaA*	CsaA protein		0.35		
PA5043	*pilN*	type 4 fimbrial biogenesis protein PilN		0.33		
PA1710	*exsC*	exoenzyme S synthesis protein C precursor		0.32		
PA5042	*pilO*	type 4 fimbrial biogenesis protein PilO		0.31		
PA1711	*exsE*	ExsE		0.28		
PA4306	*flp*	type IVb pilin, Flp		0.09	0.01	0.02
PA4303	*tadZ*	TadZ			0.50	0.37
PA4556	*pilE*	type 4 fimbrial biogenesis protein PilE			0.49	0.47
PA4304	*rcpA*	RcpA			0.28	0.29
PA0059	*osmC*	osmotically inducible protein OsmC			0.20	
PA4305	*rcpC*	RcpC			0.15	0.11
PA4527	*pilC*	pseudogene				0.50
PA0687	*hxcS*	probable type II secretion system protein				0.50
PA2675	*hplT*	probable type II secretion system protein				0.45
PA1720	*pscG*	type III export protein PscG				0.45
PA0682	*hxcX*	HxcX atypical pseudopilin				0.45
PA1382	*xqhB*	probable type II secretion system protein				0.41
PA1705	*pcrG*	regulator in type III secretion				0.35
Cell division						
PA4481	*mreB*	rod shape-determining protein MreB		2.28	10.39	13.82
PA4479	*mreD*	rod shape-determining protein MreD		2.27	3.32	2.50
PA4480	*mreC*	rod shape-determining protein MreC		2.15	3.93	2.85

PA3244	*minD*	cell division inhibitor MinD			4.20	6.62
PA5563	*soj*	chromosome partitioning protein Soj			3.08	5.30
PA3245	*minE*	cell division topological specificity factor MinE			2.45	4.89
PA3201		intracellular septation protein A			2.12	3.40
PA5564	*gidB*	glucose-inhibited division protein B			2.07	2.02
PA2615	*ftsK*	cell division protein FtsK			2.03	2.43
PA4409	*ftsQ*	cell division protein FtsQ				2.22
Nucleotide metabolism						
PA4855	*purD*	phosphoribosylamine--glycine ligase		2.27	2.30	2.37
PA4670	*prs*	ribose-phosphate pyrophosphokinase	Carbohydrate metabolism	2.25	12.01	21.84
PA1155	*nrdB*	NrdB, tyrosyl radical-harboring component of class Ia ribonucleotide reductase			6.78	14.52
PA4740	*pnp*	polynucleotide phosphorylase/polyadenylase			6.70	7.57
PA2629	*purB*	adenylosuccinate lyase	Amino acid metabolism		5.54	7.91
PA1156	*nrdA*	catalytic component of class Ia ribonucleotide reductase			5.33	9.37
PA3480	*dcd*	deoxycytidine triphosphate deaminase			4.93	7.80
PA3807	*ndk*	nucleoside diphosphate kinase			4.88	11.42
PA3637	*pyrG*	CTP synthetase			4.84	6.03
PA5298		xanthine phosphoribosyltransferase			4.66	4.79
PA3625	*surE*	stationary phase survival protein SurE			4.11	5.50
PA1013	*purC*	phosphoribosylaminoimidazole-succinocarboxamide synthase			3.86	8.23
PA5338	*spoT*	guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase			3.68	5.62
PA0342	*thyA*	thymidylate synthase			3.33	3.74
PA5426	*purE*	phosphoribosylaminoimidazole carboxylase			3.27	2.95
PA3654	*pyrH*	uridylate kinase			3.12	2.96
PA4756	*carB*	carbamoyl phosphate synthase large subunit	Amino acid metabolism		3.12	2.78
PA3770	*guaB*	inositol-5-monophosphate dehydrogenase			3.00	6.57
PA0945	*purM*	phosphoribosylaminoimidazole synthetase			2.87	2.86
PA5336	*gmk*	guanylate kinase			2.84	2.68
PA4854	*purH*	phosphoribosylaminoimidazolecarboxamide formyltransferase			2.69	2.47
PA0401	*pyrC*	dihydroorotase			2.68	3.55
PA0402	*pyrB*	aspartate carbamoyltransferase catalytic subunit	Amino acid metabolism		2.62	
PA3108	*purF*	amidophosphoribosyltransferase	Amino acid metabolism		2.56	2.39
PA4865	*ureA*	urease subunit gamma	Amino acid metabolism		2.55	4.33
PA5331	*pyrE*	orotate phosphoribosyltransferase			2.46	3.06
PA4645	*hprT*	probable purine/pyrimidine phosphoribosyl transferase			2.44	2.71
PA3763	*purL*	phosphoribosylformylglycinamidine synthase			2.42	2.16
PA0403	*pyrR*	pyrimidine regulatory protein PyrR			2.26	
PA3769	*guaA*	GMP synthase	Amino acid metabolism		2.20	2.43
PA3527	*pyrC*	dihydroorotase			2.19	2.47
PA5425	*purK*	phosphoribosylaminoimidazole carboxylase			2.11	
PA4938	*purA*	adenylosuccinate synthetase	Amino acid metabolism		2.10	2.75
PA5321	*dut*	deoxyuridine 5'-triphosphate nucleotidohydrolase			2.03	

PA4646	*upp*	uracil phosphoribosyltransferase				2.49
PA5241	*ppx*	exopolyphosphatase				2.46
PA1514		ureidoglycolate hydrolaseYbbT			0.50	0.43
PA1521		guanine deaminase			0.45	0.39
PA1515	*alc*	allantoicase			0.45	0.28
PA1523	*xdhB*	xanthine dehydrogenase			0.19	0.15
PA1524	*xdhA*	xanthine dehydrogenase			0.13	0.10
PA0441	*dht*	dihydropyrimidinase	Metabolism of other amino acids			0.31
Relication and repair						
PA3308	*hepA*	ATP-dependent helicase HepA		2.22	3.32	3.59
PA4853	*fis*			2.19	5.08	7.63
PA1804	*hupB*	DNA-binding protein HU			4.14	12.02
PA4232	*ssb*	single-strand DNA-binding protein			4.08	8.25
PA4234	*uvrA*	excinuclease ABC subunit A			3.23	3.06
PA1532	*dnaX*	DNA polymerase III subunits gamma and tau	Nucleotide metabolism		3.22	3.69
PA4042	*xseB*	exodeoxyribonuclease VII small subunit			3.19	5.87
PA3861	*rhlB*	ATP-dependent RNA helicase RhlB			3.18	4.92
PA0750	*ung*	uracil-DNA glycosylase			3.11	4.54
PA0004	*gyrB*	DNA gyrase subunit B			2.89	3.74
PA3686	*adk*	adenylate kinase	Nucleotide metabolism		2.75	6.92
PA0382	*micA*	Replication and repair and repair protein MicA			2.48	
PA4964	*parC*	DNA topoisomerase IV subunit A			2.40	
PA0001	*dnaA*	chromosomal replication initiation protein			2.31	2.88
PA0577	*dnaG*	DNA primase			2.30	2.46
PA5147	*mutY*	A / G specific adenine glycosylase			2.30	2.01
PA3620	*mutS*	Replication and repair and repair protein			2.22	
PA5332	*crc*	catabolite repression control protein			2.10	2.19
PA3011	*topA*	DNA topoisomerase I			2.10	2.18
PA0002	*dnaN*	DNA polymerase III subunit beta	Nucleotide metabolism		2.04	2.98
PA5443	*uvrD*	DNA-dependent helicase II			2.02	2.32
PA0967	*ruvB*	Holliday junction DNA helicase B			2.01	2.26
PA5493	*polA*	DNA polymerase I	Nucleotide metabolism			2.37
PA4946	*mutL*	Replication and repair and repair protein				2.26
PA0404		Holliday junction resolvase-like protein				2.24
PA5345	*recG*	ATP-dependent DNA helicase RecG				2.22
PA4931	*dnaB*	replicative DNA helicase				2.11
PA3989	*holA*	DNA polymerase III subunit delta	Nucleotide metabolism			2.01
PA3138	*uvrB*	excinuclease ABC subunit B		0.49		
PA0962		DNA-binding stress protein		0.38		
PA2738	*himA/ihfA*	integration host factor subunit alpha			0.37	0.36
PA3232		DNA polymerase III subunit epsilon	Nucleotide metabolism		0.35	
PA0720		helix destabilizing protein of bacteriophage Pf1				0.47
PA0669	*dnaE2*	error-prone DNA polymerase	Nucleotide metabolism			0.45
PA0995	*ogt*	methylated-DNA--protein-cysteine methyltransferase				0.44
Signal transduction mechanisms						
PA3192	*gltR*	two-component response regulator GltR			5.61	6.16
PA1180	*phoQ*	two-component sensor PhoQ			2.25	2.78
PA0413	*chpA*	putative chemotactic signal transduction system	Folding, sorting and degradation		2.12	2.78

PA4776	*pmrA*	PmrA: two-component regulator system response regulator PmrA				3.38
PA1179	*phoP*	two-component response regulator PhoP				2.53
PA5200	*ompR*	osmolarity response regulator				2.09
PA4546	*pilS*	two-component sensor PilS	Folding, sorting and degradation			2.03
PA1458		two-component sensor	Cell motility	0.50		
PA3702	*wspR*	two-component response regulator		0.49		
PA2571		two-component sensor		0.48	0.22	0.26
PA4296	*pprB*	two-component response regulator		0.46	0.05	0.05
PA5261	*algR*	alginate biosynthesis regulatory protein AlgR		0.29	0.09	0.13
PA2572		two-component response regulator		0.23	0.06	0.07
PA4293	*pprA*	two-component sensor PprA			0.46	0.37
PA0905	*rsmA*	regulator of secondary metabolites			0.28	0.45
PA4781		two-component response regulator			0.10	0.15
PA2808	*ptrA*	two-component response repressor, PtrA				0.32
Energy metabolism						
PA1553	*fixO/ccoO*	probable cytochrome c oxidase subunit		2.51	9.06	13.09
PA4443	*cysD*	sulfate adenylyltransferase subunit 2	Nucleotide metabolism Metabolism of other amino acids		13.99	13.61
PA5559	*atpE*	ATP synthase subunit C			12.19	55.74
PA5557	*atpH*	ATP synthase delta chain			11.89	24.28
PA5555	*atpG*	ATP synthase subunit gamma			10.68	24.06
PA5558	*atpF*	ATP synthase subunit B			9.99	34.65
PA4431		iron-sulfur protein			9.56	28.48
PA5560	*atpB*	ATP synthase subunit A			8.87	19.67
PA5556	*atpA*	ATP synthase alpha chain			8.46	13.95
PA5554	*atpD*	ATP synthase subunit beta			7.36	11.38
PA3621	*fdxA*	ferredoxin I			6.75	7.06
PA4031	*ppa*	inorganic pyrophosphatase			6.13	17.84
PA1838	*cysI*	sulfite reductase			5.22	4.95
PA5300	*cycB*	cytochrome c5			5.14	13.47
PA5561	*atpI*	ATP synthase subunit I			4.96	5.08
PA2646	*nuoK*	NADH dehydrogenase subunit K	Metabolism of cofactors and vitamins		4.37	5.09
PA2648	*nuoM*	NADH dehydrogenase subunit M	Metabolism of cofactors and vitamins		4.23	5.25
PA2639	*nuoD*	bifunctional NADH:ubiquinone oxidoreductase subunit C/D	Metabolism of cofactors and vitamins		4.17	7.61
PA4442	*cysN*	ATP sulfurylase GTP-binding subunit/APS kinase	Nucleotide metabolism Metabolism of other amino acids		4.08	3.81
PA2647	*nuoL*	NADH dehydrogenase subunit L	Metabolism of cofactors and vitamins		3.75	5.09
PA2644	*nuoI*	NADH dehydrogenase subunit I	Metabolism of cofactors and vitamins		3.44	4.42
PA2640	*nuoE*	NADH dehydrogenase subunit E	Metabolism of cofactors and vitamins		3.41	10.36
PA2645	*nuoJ*	NADH dehydrogenase subunit J	Metabolism of cofactors and vitamins		3.38	3.67
PA2641	*nuoF*	NADH dehydrogenase I subunit F	Metabolism of cofactors and vitamins		3.31	4.58
PA5490	*cc4*	cytochrome c4 precursor			3.29	7.77
PA5553	*atpC*	ATP synthase subunit epsilon			3.05	3.31
PA2952	*etfB*	electron transfer flavoprotein beta-subunit			2.80	7.21
PA2953		electron transfer flavoprotein-ubiquinone oxidoreductase			2.71	4.88

PA1482	*ccmH*	cytochrome c-type biogenesis protein CcmH			2.70	2.25
PA2649	*nuoN*	NADH dehydrogenase subunit N	Metabolism of cofactors and vitamins		2.60	2.72
PA3809	*fdx2*	ferredoxin [2Fe-2S]			2.53	2.45
PA1756	*cysH*	phosphoadenosine phosphosulfate reductase			2.48	2.35
PA3813	*iscU*	scaffold protein			2.46	
PA2637	*nuoA*	NADH dehydrogenase subunit A	Metabolism of cofactors and vitamins		2.14	2.88
PA2951	*etfA*	electron transfer flavoprotein alpha-subunit				3.79
PA2638	*nuoB*	NADH dehydrogenase subunit B	Metabolism of cofactors and vitamins			3.38
PA5242	*ppk*	polyphosphate kinase				2.63
PA1483	*cycH*	cytochrome c-type biogenesis protein				2.03
PA1479	*ccmE*	cytochrome c-type biogenesis protein CcmE		0.48		
PA2642	*nuoG*	NADH dehydrogenase subunit G	Metabolism of cofactors and vitamins	0.46	3.30	5.18
PA2643	*nuoH*	NADH dehydrogenase subunit H	Metabolism of cofactors and vitamins	0.46	2.57	3.37
PA4772		probable ferridoxin		0.42		
PA0518	*nirM*	cytochrome c-551 precursor		0.41		
PA0517	*nirC*	putative C-type cytochrome precursor		0.40		
PA0519	*nirS*	nitrite reductase precursor		0.32	2.22	2.39
PA4922	*azu*	azurin precursor		0.30		2.24
PA3930	*cioA*	cyanide insensitive terminal oxidase		0.28	0.09	0.20
PA1172	*napC*	cytochrome c-type protein NapC		0.22	0.11	0.22
PA1174	*napA*	nitrate reductase		0.17	0.08	0.19
PA1176	*napF*	ferredoxin protein NapF		0.17	0.05	0.11
PA1551	*fixG*	probable ferredoxin		0.17		
PA1175	*napD*	NapD protein of periplasmic nitrate reductase		0.14	0.08	0.14
PA1173	*napB*	cytochrome c-type protein NapB precursor		0.14	0.07	0.18
PA3331		cytochrome P450		0.12	0.35	
PA1555		cytochrome c		0.10	7.36	8.57
PA1557		cytochrome oxidase subunit (cbb3-type)		0.09	2.89	3.21
PA4130		sulfite or nitrite reductase		0.08	0.26	0.43
PA1177	*napE*	periplasmic nitrate reductase protein NapE		0.08	0.02	0.04
PA1556		cytochrome c oxidase subunit		0.06	7.92	10.46
PA4587	*ccpR*	cytochrome c551 peroxidase precursor		0.05		2.02
PA4133		cytochrome c oxidase subunit (cbb3-type)		0.03	0.14	
PA3929	*cioB*	cyanide insensitive terminal oxidase			0.35	
PA3032	*snr1*	cytochrome c Snr1			0.11	0.21
PA0108	*coIII*	cytochrome c oxidase, subunit III			0.06	0.16
PA0107		cytochrome c oxidase assembly protein			0.05	0.12
PA0105	*coxB*	cytochrome c oxidase, subunit II			0.04	0.12
PA0106	*coxA*	cytochrome c oxidase, subunit I			0.04	0.11
PA2514	*antC*	anthranilate dioxygenase reductase	Carbohydrate metabolism			0.21
PA2512	*antA*	anthranilate dioxygenase large subunit	Carbohydrate metabolism			0.08
PA2513	*antB*	anthranilate dioxygenase small subunit	Carbohydrate metabolism			0.04
Glycan biosynthesis and metabolism						
PA3147	*wbpJ*	glycosyl transferase WbpJ		2.09	4.96	5.43
PA1812	*mltD*	membrane-bound lytic murein transglycosylase D precursor			5.30	10.68
PA4545	*comL*	competence protein ComL			4.70	7.43
PA3149	*wbpH*	glycosyltransferase WbpH			4.57	4.57

PA3644	*lpxA*	UDP-N-acetylglucosamine acyltransferase			4.22	6.21
PA3150	*wbpG*	LPS biosynthesis protein WbpG			4.17	5.06
PA3999	*dacC*	D-ala-D-ala-carboxypeptidase			4.13	5.55
PA3646	*lpxD*	UDP-3-O-[3-hydroxylauroyl] glucosamine N-acyltransferase			3.96	4.69
PA3158	*wbpB*	oxidoreductase WpbB			3.61	4.36
PA0705	*migA*	alpha-1,6-rhamnosyltransferase MigA			3.54	3.32
PA3146	*wbpK*	NAD-dependent epimerase/dehydratase WbpK			3.51	3.17
PA4425	*gmhA*	sedoheptulose 7-phosphate isomerase GmhA			3.25	6.74
PA3155	*wbpE*	aminotransferase WbpE			3.20	5.22
PA0766	*mucD*	serine protease MucD precursor			3.08	4.87
PA5276	*lppL*	lipopeptide LppL precursor			2.73	6.91
PA3643	*lpxB*	lipid-A-disaccharide synthase			2.71	2.33
PA2966	*acpP*	acyl carrier protein			2.63	4.31
PA4700	*mrcB*	penicillin-binding protein 1B			2.63	2.96
PA1959	*bacA*	undecaprenyl pyrophosphate phosphatase			2.56	2.99
PA4001	*sltB1*	soluble lytic transglycosylase B			2.47	3.11
PA4003	*pbpA*	penicillin-binding protein 2			2.24	2.40
PA3159	*wbpA*	UDP-glucose/GDP-mannose dehydrogenase WbpA			2.38	4.70
PA4406	*lpxC*	UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase			2.21	3.74
PA0011		lipid A biosynthesis lauroyl acyltransferase			2.16	2.21
PA4996	*rfaE*	LPS biosynthesis protein RfaE			2.10	
PA0765	*mucC*	positive regulator for alginate biosynthesis MucC			2.06	2.00
PA1222	*mltA*	probable membrane-bound lytic murein transglycolase A			2.00	
PA4947	*amiB*	N-acetylmuramoyl-L-alanine amidase				3.03
PA5045	*ponA*	penicillin-binding protein 1A				2.90
PA3156	*wbpD*	acetyltransferase WbpD				2.62
PA1792	*ybbF*	UDP-2,3-diacylglucosamine hydrolase				2.13
PA4415	*mraY*	phospho-N-acetylmuramoyl-pentapeptide-transferase				2.00
PA3141	*wbpM*	nucleotide sugar epimerase/dehydratase WbpM		0.39		
PA3337	*rfaD*	ADP-L-glycero-D-mannoheptose 6-epimerase		0.24		
PA0763	*mucA*	anti-sigma factor MucA	Translation		0.06	0.05
PA1385		glycosyl transferase				0.41
Cell motility						
PA4959	*fimX*	FimX			2.64	3.31
PA4953	*motB*	flagellar motor protein MotB			2.01	3.43
PA1092	*fliC*	flagellin type B	Signal transduction	0.47	0.33	0.35
PA3351	*flgM*	FlgM		0.47	0.30	0.22
PA3708	*wspA*	putative chemotaxis transducer		0.46		
PA4310	*pctB*	chemotactic transducer PctB		0.46		
PA4307	*pctC*	chemotactic transducer PctC		0.39		
PA4309	*pctA*	chemotactic transducer PctA		0.36	0.44	
PA1094	*fliD*	flagellar capping protein FliD		0.35	0.26	0.23
PA0176	*aer2*	aerotaxis transducer Aer2		0.28	0.15	0.32
PA2573		chemotaxis transducer		0.25	0.02	0.02
PA1561	*aer*	aerotaxis receptor Aer	Signal transduction	0.22	0.37	0.43

PA2128	*cupA1*	fimbrial subunit CupA1		0.15		2.33
PA1454	*fleN*	flagellar synthesis regulator FleN			0.49	0.44
PA1453	*flhF*	flagellar biosynthesis regulator FlhF			0.45	0.34
PA1095	*fliS*	flagellar protein FliS			0.44	0.32
PA1087	*flgL*	flagellar hook-associated protein FlgL			0.41	0.40
PA1423	*bdlA*	BdlA			0.27	0.23
PA4915		chemotaxis transducer			0.19	0.24
PA3361	*lecB*	fucose-binding lectin PA-IIL			0.16	0.50
PA2570	*lecA*	LecA	Adaptation, protection LPS		0.10	0.35
PA3350	*flgA*	flagellar basal body P-ring biosynthesis protein FlgA				0.50
PA1083	*flgH*	flagellar basal body L-ring protein				0.49
PA1444	*fliN*	flagellar motor switch protein	Folding, sorting and degradation			0.45
PA1100	*fliE*	flagellar hook-basal body protein FliE				0.27

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